Assessment of angiogenesis as an early predictor of response to primary endocrine therapy in breast cancer.

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To my parents, for their love and support,
To Stuart, for his patience and understanding,
To Archie and Iona, for their smiles.

"Those who hope in the Lord will renew their strength.
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint."
Isaiah 40: 31

I confirm that this thesis has been composed by myself.

I acknowledge that the work reported was done by myself at the Edinburgh Breast Unit, where I have worked for the past two years. Exceptions to this are VEGF staining which was carried out by Dr. J. Harmey of the Royal College of Surgeons of Ireland, MIB-1 staining carried out by Lisa MacFarlane of the Breast Unit Research Group and MIB-1 assessments, carried out by Miss. S. Iqbal of the Breast Unit Research Group, Edinburgh. I am however indebted to several people for advice, instruction and assistance in the preparation of materials, as acknowledged.

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Contents

Abstract

Anti-oestrogens, such as tamoxifen, are effective agents in the treatment of breast cancer, reducing recurrence and death with minimal side effects. However, a substantial number of tumours fail to respond and mechanisms of response and resistance are poorly understood. Angiogenesis, or new vessel formation, is an essential component of tumour growth and metastasis. Oestrogens act to preserve endothelial cells by inhibiting their apoptosis. Conversely, tamoxifen exerts an anti-angiogenic effect. Changes in tumour vascularity during primary tamoxifen treatment may thus provide an early marker of hormone sensitivity.

The aims of this study were to determine whether changes in tumour vascularity followed primary treatment with tamoxifen, to ascertain whether such changes were associated with tumour response and to define chronology of such changes. Potential methodological issues such as observer variation and reproducibility of counts were addressed. Finally, a study was performed to determine whether changes in tumour vascularity provided a useful predictor of sensitivity to primary endocrine therapy.

Investigation of observer variation yielded low rates of both inter- and intra-observer variation and good correlation between counts performed following staining with Factor VIII and CD31 antibodies. The study undertaken to address reproducibility of microvessel counts in different breast cancer specimens demonstrated that, although counts were similar in core biopsies and sections taken from the same tumour, no significant positive correlation was found. This suggested that there might be difficulty in making comparisons within tumours unless the effects of treatment on vascularity were large.

Fifty-seven patients with large, oestrogen receptor (ER)-positive breast cancers were treated with tamoxifen prior to surgery. Tumour vascularity was assessed before and after 3 months' treatment and changes correlated with response. A significant association was found between change in tumour vascularity and response, with microvessel counts decreasing in responding tumours ($p=0.006$) and tending to increase in non-responding tumours ($p=0.036$). Thus successful treatment with tamoxifen resulted in reduced tumour vascularity. Immunohistochemical staining for vascular endothelial growth factor (VEGF) was studied in the same group of tumours and demonstrated a significant reduction in proportion of tumour cells expressing VEGF in responding tumours.

To determine timing of these changes in relation to the effect on tumour size, mice bearing xenografts of ER-positive ZR-75 and ER-negative MDA-MB-231 breast cancer cell lines were treated with tamoxifen and assessed for changes in tumour size and vascularity. After 2 days, there was evidence of reduced microvessel counts in ZR-75, but not MDA-MB-231 tumours. Clear evidence of tumour regression in ZR-75 tumours only became apparent after 4 days. These results suggested that changes in vascularity precede tumour regression.

Forty-two patients were recruited into a prospective study in which patients were treated with primary tamoxifen. Tumour tissue in the form of core biopsies was available prior to and after fourteen days of treatment. Patients underwent definitive surgery at the end of three months. Microvessel counts were compared at the three time points. The only significant difference identified between non-responding and responding tumours was increased microvessel counts in non-responding tumours following two weeks of treatment ($p=0.01$). No significant trends were observed at three months. Changes in proliferation during treatment with tamoxifen were studied in the same group of patients for comparison. There was a significant trend for reduction in proliferation in responding tumours which was maintained throughout the period of treatment.

Response to primary treatment with tamoxifen was associated with reduced tumour vascularity and xenograft data suggested that such changes occur prior to tumour regression. However, problems with reproducibility of microvessel counts in small tumour biopsies may limit their role as an early predictor of sensitivity to endocrine therapy. Preliminary results suggest that early changes in proliferation may aid in the prediction of endocrine sensitivity.

Introduction

Incidence of breast cancer:

Breast cancer is primarily a disease of women with only 1% of cases occurring in men. It poses an important international health care problem, causing immeasurable morbidity and substantial mortality and economic consequences throughout the world. The age-standardised incidence rates of breast cancer in the United Kingdom, North America, Australasia and Scandinavia are approximately 60 to 70 per 100 000 women per annum (Parkin *et al*, 1992). In the UK alone, 25 000 new cases are diagnosed and 15 000 women die from breast cancer each year (Forrest, 1987). It is the commonest cancer to affect women in Scotland (Sharp *et al*, 1993). A third of this risk occurs after the age of 75, with a 1 in 15 risk quoted for women up to the age of 75 (Sharp *et al.*, 1993). Furthermore there is evidence that the incidence is increasing, both in Scotland (Sharp *et al*, 1993) and in the US (Feuer *et al*, 1993). By the year 2000, breast cancer will affect approximately one million women and cause the death of over 400 000 women per annum (Forbes, 1997). This increase may be due in part to increased detection, following commencement of the National Screening Programme, and increased longevity. Despite taking these factors into account, there remains a steady increase, which may caused by dietary or environmental factors.

Aetiology:

Although the cause of breast cancer remains unknown, there are a number of factors that affect risk of developing the disease. It is predominantly a disease of women, living in the Western world (Forrest, 1997). The incidence rises steadily with age, with one third of breast cancers being diagnosed after the age of 75 (Henderson, 1990).

Hormonal factors:

Functioning ovaries are a pre-requisite for the initiation of breast cancer; women who undergo oophorectomy before the age of 35 years reduce their risk by more than half (Trichopoulos *et al*, 1972). Women in whom reproductive life is extended, by early menarche or late menopause, have an increased risk (MacMahon *et al*, 1982). Reproductive factors associated with breast cancer risk include older age at first birth and of subsequent births. There is a dual effect of pregnancy, a transient period of increased risk for the three years post-delivery is followed by a longer term protective effect (Leon *et al.*, 1995). There is probably no increased risk with induced abortion (Melbye *et al*, 1997) and a weak protective effect of breastfeeding (Thomas & Noonan, 1993).

Genetic factors:

There is no doubt that for some women there is an increased risk of breast cancer related to the occurrence of disease in other members of the same family. The risk of developing the disease can be two to five times higher than the normal population if one or more first or second degree relatives have been affected (Anderson & Badzioch, 1985). There is evidence to suggest that in families with very high risk of developing breast cancer, an inherited autosomal dominant gene is responsible (Lynch *et al*, 1984). Between 5 and 9% of all breast cancers are due to hereditary causes (Lynch *et al.*, 1992), and inherited factors are thought to contribute to approximately 25-35% of cases diagnosed before the age of 30 years (Lynch *et al.*, 1992). Mutations in BRCA-1 (Miki *et al*, 1994) or BRCA-2 genes (Wooster *et al*, 1995) result in a high number of inherited forms of breast cancer. The BRCA-1 gene on chromosome 17q was identified by positional cloning, and consists of 22 coding exons distributed over 100kb of genomic DNA, encoding a protein of 1863 amino acids (Miki *et al.*, 1994). Evidence suggests linkage to BRCA-1 in families with early onset breast or

ovarian cancer, or both, occurring in multiple generations (Ford & Easton, 1995). The cumulative risk of breast cancer development in BRCA-1 carriers is estimated to be in the range of 55-85%. Carriers may also carry increased risk of developing colon and prostate cancer (Ford & Easton, 1995). Gene frequency studies of BRCA-1 suggest a frequency of 1:2-400 in the general population (Biesecker *et al*, 1993), with a higher frequency in Ashkenazi Jews (Offit *et al*, 1996). Studies of this group suggest that one fifth of breast cancers occurring before the age of 50 are due to BRCA-1 mutations (Offit *et al*., 1996). Estimates of lifetime risk are based on selected families with high incidence and so should be considered upper limits.

The BRCA-2 gene is located on chromosome 13q12-13 and is also associated with early onset breast cancer (Wooster *et al*, 1995). Linkage to the BRCA-2 gene is suggested when male breast cancer occurs in the family. The cumulative risk among carriers is estimated to be 63% by age 70 and 87% by age 80. A higher proportion of breast cancer cases may be due to low penetrant susceptibility genes that are more common, such as HRAS1 and ataxia-telangectasia (Ford & Easton, 1995).

Environmental factors:

Several lifestyle factors contribute to the risk of developing breast cancer. An association has been found between recent stressful life events and risk (Ginsberg *et al*, 1996). There has been much work observing effects of diet on risk, and there is evidence to suggest that diet rich in fat leads to increased risk (Lubin *et al*, 1986). This is contentious, and a recent meta-analysis suggested that there might be no such increase (Hunter *et al*, 1996). It may be that dietary fat is particularly important during youth, and that, if consumed excessively during this period, it may lead to a slight increased risk in developing breast cancer (Hunter & Willet, 1996). The type of dietary fat is important, olive oil (Hunter & Willet, 1996) and milk (Knekt *et al*, 1996) have a protective effect. Other dietary constituents that may exert a protective effect are the phytoestrogens, which are found in high concentrations in soya products (Knight & Eden, 1995), and vitamin A (Hunter & Willet, 1996).

Smoking has not been found to significantly increase risk of developing the disease (Palmer & Rosenberg, 1993), although most studies have ignored the possible effects of passive smoking. When this is taken into account, significant risks were observed for both passive and active smoking (Morabia *et al*, 1996). Alcohol consumption of more than three units a

day increases breast cancer risk, particularly in women over 50 years (Holmberg *et al*, 1995). Regular exercise for longer than 3.5 hours a week appears to have a protective effect (Thune *et al*, 1997), although a recent study examining the effect of exercise in late adolescence and in the recent past failed to identify such an effect (Rockhill *et al*., 1998).

Th effect of exogenous hormones has been widely investigated both in the context of oral contraceptive preparations (OCP) and hormone replacement therapy (HRT). In current users of OCP there is a slight increased risk of developing breast cancer (relative risk 1.24) when compared with never users. This increased risk ceases 10 years after stopping the pill (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Other features of OCP use identified as risk factors for breast cancer in previous studies such as duration of use (UK National Case-Control Study Group, 1989), age at first use (Pike *et al*, 1983) and dose and type of OCP (Pike *et al*, 1983) have little effect when compared with current use.

A recent review of literature concerning risk of breast cancer in association with HRT use has demonstrated an increasing risk per year of HRT use which is equivalent to delaying menopause by a year (Colditz, 1998). There is a positive relationship between hormone levels and risk, and risk increases with increasing duration of use (Colditz, 1998). Whether this increased risk is due to regular surveillance of women on HRT, leading to detection bias, or is a true biological effect is not known, although an increasing body of evidence suggests the risk to be true. However, this must be balanced against the benefits of HRT to the cardiovascular system (Stampfer *et al*, 1991) and to bone metabolism (Lindsay *et al*, 1976).

The Normal Breast:

Some discussion of development of the normal breast is relevant in understanding the role of oestrogen in breast cancer and thus the rationale behind use of hormonal therapeutic strategies. The breast is a secretory organ, composed of glandular tissue and ducts contained in a stroma of supporting fat and fibrous tissue. The primary secreting units consist of groups of terminal ductules with sac-like ends, embedded in a fine specialized connective tissue to form breast lobules, or terminal ductal lobular units (TDLU). During lactation these become dilated to form alveoli which secrete milk. A coalescing system of ducts of gradually increasing size drain the TDLU, ending in 12 to 15 main lactiferous ducts which emerge on the surface of the nipple. With the exception of the main lactiferous ducts, the whole of the duct system is lined by a single layer of epithelial cells. It is from the luminal epithelial cells of TDLUs that most pre-invasive and invasive cancers arise (Wellings *et al*, 1975).

Normal breast development is characterised by three phases of activity: puberty, ovulatory menstrual cycles and pregnancy. Oestrogen is required for breast development in puberty: in girls whose breasts do not form because of gonadal dysgenesis, full development may be induced by administration of oestrogen (Laron *et al*, 1989), but oestrogen alone is not sufficient (Bidlinggmaier & Knorr, 1978). Several other factors, such as gonadotrophins, glucocorticoids and growth factors are likely to play a role (Miller, 1996c). Conversely, menopause is associated with breast involution; epithelial cell proliferation decreases with age (Potten *et al*, 1988), and glandular epithelium is replaced by fat. The timing of such changes corresponds to a reduction in circulating oestrogens.

The breast responds to the cyclical nature of hormone secretion during the menstrual cycle. The luminal epithelial cells of TDLU undergo greater proliferation during the luteal phase, during which period levels of circulating oestrogen and progesterone are moderately elevated (Anderson *et al*, 1982). Oestrogen levels are at their maximum during the follicular phase. Effects of oestrogen are mediated by oestrogen receptors (ER), which are expressed in normal breast in lower levels than in cancer (Carpenter *et al*, 1989), with varying levels of expression throughout the menstrual cycle. Lowest levels are expressed during the luteal phase (Battersby *et al*, 1992), coinciding with maximum levels of circulating oestrogen. A picture emerges of a constantly changing hormonal milieu resulting in changes in cell proliferation and gene expression throughout the menstrual cycle.

Management of breast cancer:

For the purpose of treatment, breast cancer can be divided into two categories: early, stage I and II disease, which is considered operable, and advanced or inoperable, stage III and IV disease. The aims of treatment differ between the two groups: in early breast cancer aims are to achieve local control and treat micrometastatic disease, with the ultimate goal of cure, in advanced disease aims are to control disease whilst maintaining quality of life. Management of two areas must be considered in early breast cancer: locoregional and systemic disease. In advanced breast cancer, management of locoregional disease is not aimed to cure, but to palliate, so effects of interventions on quality of life must be considered.

Management of locoregional disease:

The mainstay of locoregional treatment of early breast cancer is surgery to the breast and axilla. There has been a change in pattern of breast surgery over recent decades. Radical surgical resection has been supplanted by breast conservation surgery (Veronesi *et al*, 1981). This change can be attributed to several factors: firstly, a greater understanding that long term outcome following breast cancer treatment is influenced more by systemic than locoregional therapies has encouraged surgeons to consider less radical options (Fisher & Gebhardt, 1978). Secondly, changing patterns in presentation of breast cancer have followed the advent of mammographic screening, resulting in earlier diagnosis. Thus an increasing number of cancers are diagnosed when they are less than 1cm in diameter or at the pre-invasive stage (Cady, 1997). Finally, patients are increasingly expressing a wish to undergo conservative surgery with resultant organ preservation and improved cosmesis.

Trials comparing results of mastectomy and conservative surgery have shown similar rates of overall survival, but significantly increased local recurrence rates in those undergoing conservative surgery without postoperative radiotherapy (Fisher *et al*, 1985). In the large National Surgical Adjuvant Breast and Bowel Project (NSABP) B-06 study with 12 years of follow up, the cumulative incidence of recurrence in the ipsilateral breast was 35% in patients treated with lumpectomy alone versus 10% in the group treated with lumpectomy and breast irradiation ($p<0.001$) (Fisher *et al*, 1995). An important predictor of local recurrence following conservative surgery is involvement of surgical margins (Smitt *et al*, 1995; Veronesi *et al*, 1990). The observed lack of difference in survival between the two groups was thought to be due to a balance between the slight reduction in breast cancer

deaths and an increase in the number of cardiac related deaths in the radiotherapy-treated group (Early Breast Cancer Trialists' Collaborative Group, 1995). However, more recent trials have confirmed lower mortality from breast cancer and have also suggested a reduction in cardiac related deaths, possibly due to reduced dosage in radiotherapy regimens (Cuzick *et al*, 1994).

Breast conservation has thus become the treatment of choice for patients with a single clinical and/or mammographic lesion measuring 4cm or less without signs of local advancement, extensive nodal involvement or metastases. Patients with central lesions, multicentric disease, extensive ductal carcinoma in situ or large tumours in small breasts are ineligible (Dixon & Sainsbury, 1998).

Another important aspect of surgical management of breast cancer is the axilla. The changing pattern of presentation and treatment of breast cancer described above has led to debate over the necessity of complete (level III) axillary dissection in all patients. The role of axillary surgery is twofold: to stage the axilla and to treat established disease. In the 1980s, physicians based decisions regarding administration of adjuvant systemic therapy on pathologic status of axillary lymph nodes, leading to an increasing number of patients undergoing axillary dissection, although results did not affect subsequent management in all patients (Haffty *et al*, 1998). In those patients with involved nodes, axillary dissection is the optimal method of treatment but it carries risks of complications including lymphoedema and damage to nerves of the axilla. Prediction of nodal involvement prior to undertaking surgery would therefore be valuable. There is a direct relationship between tumour size and presence of lymph node metastases; those with mammographically detected disease are less likely to have involved nodes at each individual size. Thus such patients with small tumours may undergo sampling instead of dissection with reduced morbidity (Dixon & Sainsbury, 1998). Patients undergoing mastectomy should undergo axillary dissection to reduce the need for postoperative radiotherapy (Dixon & Sainsbury, 1998).

Alternative options for management of the axilla are node sampling or sentinel node biopsy. Axillary node sampling, involving excision of four nodes, has been compared to level III dissection in patients undergoing mastectomy for breast cancer: patients with positive lymph nodes underwent axillary radiotherapy postoperatively. There was a similar rate of detection of positive lymph nodes and similar rates of relapse-free and overall survival in the two

groups (Forrest *et al*, 1995). Radiotherapy reduced arm mobility and axillary dissection increased rate of arm swelling (Aitken *et al*, 1989).

The sentinel lymph node is the first node draining the primary tumour in the regional lymphatic basin and its histologic nature may predict characteristics of the remaining axillary lymph nodes (Albertini *et al*, 1996). Sentinel node biopsy is a recently developed technique, involving injection of radiolabelled sulphur colloid or albumin, blue dye or a combination into the tumour and skin overlying it, allowing identification of the sentinel node by radiation monitoring or intraoperative visualization. In one study using a combination of mapping techniques and sentinel node biopsy followed by axillary clearance, the sentinel node was successfully identified in 92% of patients; 32% were found to have metastatic disease, the sentinel node biopsy tested positive in all these cases (Albertini *et al*, 1996). Accurate assessment of sentinel nodes intraoperatively may prevent unnecessary axillary dissection.

Breast biopsy techniques are an important consideration in the management of locoregional breast cancer. The use of fine needle aspiration cytology is well-established as part of the triple assessment required for the accurate diagnosis of breast cancer, with a sensitivity of 97% and false negative rate of 3% in one study of screen detected cancers (Yiangou *et al*, 1996). Core biopsies provide histological information and are being used increasingly in the diagnostic setting, with improved results of detection of invasive and pre-invasive cancers (Litherland *et al*, 1996). Core biopsies are important in the setting of advanced disease, providing the only tissue available prior to commencement of systemic therapy or radiotherapy (Pace & Berg, 1997). Assessment of cores can be performed to determine prognostic markers, such as oestrogen receptor status, that may influence choice of primary treatment (Zidan *et al*, 1997). However, reliability of core biopsy specimens in such assessments requires confirmation because of their relatively small size and tumour heterogeneity. One study has demonstrated concordance between core and tumour section in several pathological variables including oestrogen receptor, bcl-2 and p53 (Jacobs *et al*, 1998). In the same study, assessment of microvessel density in core biopsies following staining with Factor VIII antibody was not found to be reliable (Jacobs *et al*, 1998).

Management of systemic disease:

Adjuvant systemic therapy for early breast cancer began in the early 1970s when it was first appreciated that loco-regional treatment was frequently insufficient to control disease, leading to the suggestion that occult micrometastases were present at diagnosis (Valagussa *et al*, 1978). Whilst benefits of such therapy has been shown in several clinical trials (Early Breast Cancer Trialists' Collaborative Group, 1992; Olivotto *et al*, 1994), its effect on individual patients cannot be assessed as there is no overt disease to monitor as treatment follows primary tumour excision. Adjuvant systemic therapy may involve administration of chemotherapy or endocrine therapy or a combination of both. In an overview from the Early Breast Cancer Trialists' Collaborative Group, chemotherapy was found to reduce annual risk of death by 30% in premenopausal patients and was most effective in younger patients with ER-negative tumours (Early Breast Cancer Trialists' Collaborative Group, 1992). There was a less marked survival improvement in postmenopausal women given adjuvant chemotherapy (Early Breast Cancer Trialists' Collaborative Group, 1992). There is a wide variety of chemotherapy regimens available, which is beyond the scope of this thesis, the main focus of which is endocrine treatment of breast cancer.

Endocrine treatment of breast cancer:

It was Beatson who, in 1896, first noted that ovarian ablation in premenopausal women with advanced breast cancer resulted in tumour regression (Beatson, 1896). It has become clear that such regression occurred in approximately one-third of patients with advanced disease following oophorectomy (Thomson, 1902). Adjuvant intervention in premenopausal women results in a reduction in odds ratio of death of 28% when compared to women not receiving adjuvant therapy (Early Breast Cancer Trialists' Collaborative Group, 1992). A Scottish study compared survival rates in premenopausal women with lymph node positive disease treated in the adjuvant setting with either ovarian ablation or chemotherapy. No significant differences in relapse-free or overall survival were observed between the two treatments (Scottish Cancer Trials Breast Group & ICRF Breast Unit, 1993). Other methods of surgical endocrine manipulation, such as hypophysectomy and adrenalectomy, have also been shown to be of benefit. Such manipulations produce benefits in 30-40% of postmenopausal women with advanced breast cancer (West *et al*, 1952), but these are associated with high morbidity and a need for replacement corticosteroid therapy. This has led to the development of more specific drug therapies to produce hormone deprivation.

Advantages of drug therapy are that it is non-invasive and reversible, so that if endocrine manipulation fails, the drug can be stopped with cessation of side-effects. LHRH agonist analogues effect a 'medical oophorectomy', by suppressing oestrogen production in premenopausal women (Nicholson *et al*, 1987). LHRH is a decapeptide, intermittently secreted by the hypothalamus, which, via release of gonadotrophins, stimulates ovarian oestrogen production. By maintaining continuous occupancy of specific receptors for LHRH, LHRH agonist analogues cause a fall in oestrogen production (Nicholson & Walker, 1989). Goserelin (Zoladex), an LHRH agonist, is administered monthly by depot injection and can be as effective as surgical or radiotherapeutic ovarian ablation with response rates of up to 40% in women with advanced tumours (Santen *et al*, 1990). This increases to 50% in women whose tumours are selected based on oestrogen receptor positivity (Santen *et al*, 1990).

Early development of antioestrogens was based on the rationale that oestrogen exerts its effects via specific receptors and that disruption of such interaction would produce tumour regression. Tamoxifen is a partial oestrogen agonist which competes with oestrogen for binding to the oestrogen receptor, blocking the effect of endogenous oestrogen, although it exerts a weak agonist effect particularly at low levels of endogenous oestrogen (Jordan &

Murphy, 1990). Tamoxifen was first used in palliation of advanced disease: in a study of 4000 women treated with the drug, response was observed in one-third of women (Jackson & Lowery, 1987). Response rates were higher in women with ER-positive disease (Hawkins & Roberts, 1980). The percentage of ER-positive tumours increases with age independent of menopausal status, so that elderly women tend to have higher response rates than younger women (Bradbeer & Kyngdon, 1983). These factors, combined with the fact that tamoxifen is well-tolerated with few side effects, led to its establishment as first line therapy in those elderly women for whom surgery is associated with a significantly greater risk of perioperative morbidity and mortality (Miller, 1996b).

Before exploring the use of tamoxifen in further detail, other endocrine therapies will be mentioned. Work in this thesis is based primarily on treatment of breast cancers with tamoxifen so that emphasis will be placed on this, but other drugs warrant a brief outline. Problems arising from the agonist effects of tamoxifen include increased incidence of endometrial and hepatic tumours (Jordan & Murphy, 1990), which has led investigators to explore alternative methods of ER blockade. Pure antiestrogens have been found to be more effective than tamoxifen in cell culture (Wakeling & Bowler, 1989) and clinical studies suggest that they produce benefit as second line hormonal therapy following development of tamoxifen resistance (De Friend *et al*, 1994).

An alternative strategy to ER blockade for combating oestrogen-stimulated tumour growth is by the inhibition of oestrogen synthesis. The final step in oestrogen production is conversion of androgens to oestrogen, catalysed by the aromatase enzyme. Understanding of this process has led to development of specific aromatase inhibitors (Harvey, 1998). The first to be used was aminoglutethimide, which induced a clinical response in 33% of patients with advanced disease, with a median duration of response of 18 months (Griffiths *et al*, 1973). Aminoglutethimide lacks specificity and is therefore associated with significant side effects, requiring concomitant corticosteroid administration (Miller, 1996b). The more recent development of new specific aromatase inhibitors, such as letrozole and anastrozole, has led to decreased toxicity and increased efficacy, leading to tumour remission in postmenopausal patients (Goss & Gwyn, 1994).

Role of tamoxifen in breast cancer:

The role of tamoxifen is breast cancer management can be divided into two categories: treatment of established disease, which may be early or advanced, and prophylaxis for patients at high risk of developing the disease.

Adjuvant therapy:

The aim of adjuvant therapy for patients with early breast cancer is to eradicate or control occult metastases without significantly worsening quality of life. In a large overview of 37 000 women with early breast cancer treated with 5 years of adjuvant tamoxifen following surgery, proportional recurrence reductions of 47% and mortality reductions of 26% were achieved compared with women who received no adjuvant therapy at 10 years follow up (Early Breast Cancer Trialists' Collaborative Group, 1998). Reductions in recurrence rates were most evident in the first five years, with main benefits in reducing mortality continuing to increase up to 10 years after treatment (Early Breast Cancer Trialists' Collaborative Group, 1998). Absolute benefit was greater in women with lymph node-positive rather than lymph node-negative disease, and treatment was more effective in women over the age of 50 years (Early Breast Cancer Trialists' Collaborative Group, 1998). A strong indicator of benefit was ER status, with minimal benefit in those patients with ER-negative tumours (Early Breast Cancer Trialists' Collaborative Group, 1998), contrary to early evidence (Early Breast Cancer Trialists' Collaborative Group, 1992).

Other studies investigating the role of tamoxifen have attempted to determine the place of combined chemoendocrine therapy in the adjuvant setting. Premenopausal women with lymph node-positive, ER-positive disease derived some benefit from such combined therapy, with improved relapse-free and overall survival (Fisher *et al*, 1997; Fisher *et al*, 1981). Benefits of adjuvant tamoxifen were greatest for patients over the age of 50 years (Fisher *et al*, 1981). Addition of chemotherapy led to a modest improvement in relapse-free survival (International Breast Cancer Study Group, 1997), but a meta-analysis of quality-adjusted survival found that chemoendocrine therapy did not provide more quality-adjusted survival time when compared to tamoxifen alone (Gelber *et al*, 1996).

Optimal duration of treatment with adjuvant tamoxifen remains controversial. Early studies involved treatment with tamoxifen for up to two years (Early Breast Cancer Trialists'

Collaborative Group, 1992). Improved understanding of the action of tamoxifen as a cytostatic agent led to the suggestion that stopping treatment may lead to early relapse. This has provoked several studies aimed at determining the optimal duration of adjuvant tamoxifen (Swedish Breast Cancer Cooperative Group, 1996). Comparison of relapse-free (RFS) and overall survival (OS) in patients treated with 2 versus 5 years of tamoxifen showed continued benefits from longer duration of treatment, with improved relapse-free and overall survival (Swedish Breast Cancer Cooperative Group, 1996). The controversy over 5 years versus longer continues (Rea *et al*, 1998). Results from the NSABP-B-14 trial suggested that continuing tamoxifen beyond 5 years in patients with primary operable, lymph node-negative, ER-positive tumours was detrimental in terms of RFS, distant disease-free survival and OS compared with those who stopped at 5 years (Fisher *et al*, 1996). Increased rates of thromboembolic episodes and endometrial cancer were observed in the treated group (Fisher *et al*, 1996). Tormey et al found that the only group to benefit from continued tamoxifen beyond five years were those patients with ER-positive disease, who experienced a longer time to relapse (Tormey *et al*, 1996). Results from the Scottish trial suggested that a worthwhile gain from continuing adjuvant tamoxifen beyond five years is unlikely (Stewart *et al*, 1996). Two major trials aimed at definitively answering the question of optimum duration of adjuvant tamoxifen are ongoing (Rea *et al*, 1998).

Treatment of advanced disease:

Tamoxifen was first evaluated in the setting of advanced disease in postmenopausal women (Cole *et al*, 1971). An early review of 1200 patients with advanced disease described a response rate to tamoxifen of 32% with improved survival in responding patients, compared with non-responders (Mouridsen *et al*, 1978). Response rates rose to greater than 50% in ER-positive patients (Kiang & Kennedy, 1977), and in patients who had previously responded to other forms of endocrine therapies (Kiang & Kennedy, 1977). Remission rates were found to be higher in patients with soft tissue or lung metastases than in those with bony metastases, which responded better to other forms of endocrine treatment such as medroxyprogesterone (Van Veelen *et al*, 1986). When compared with other forms of hormonal manipulation, tamoxifen favoured well with similar response rates and duration of response, but with considerably fewer side effects (Ingle *et al*, 1981; Van Veelen *et al*, 1986). Recent studies comparing the efficacy of the new generation aromatase inhibitors have yielded interesting results: they appear to be as effective as tamoxifen with potential for superior tolerability (Anderson *et al*, 1998; Goss & Gwyn, 1994).

Tamoxifen also plays a role in the treatment of premenopausal women with advanced breast cancer. Studies have shown a remission rate of approximately 30% with a median duration response of 13 months (Margreiter & Wiegele, 1984). Tamoxifen compared favourably with surgical oophorectomy, with similar response rates, median duration of response and overall median survival (Buchanan *et al*, 1986). Tamoxifen was better tolerated and so provides a suitable alternative to surgical oophorectomy in these patients (Buchanan *et al*, 1986).

Tamoxifen as prophylaxis:

The potential role of tamoxifen in the prevention of breast cancer developed as a result of finding that tamoxifen reduced risk of contralateral cancer by 39% (Early Breast Cancer Trialists' Collaborative Group, 1992). The effect was found to increase with duration of tamoxifen treatment. The subject remains contentious and most large multicentre studies are ongoing. The NSABP study aimed at addressing this issue was closed earlier than planned this year because of finding a 45% reduction in healthy women given tamoxifen versus placebo (Stat-Bite, 1998). European trials continue as such clear benefits have not been observed (Powles *et al*, 1998).

Neoadjuvant therapy:

Neoadjuvant therapy, or administration of systemic therapy prior to loco-regional therapy, has been used increasingly in the management of breast cancer since the 1970s (Forrest *et al*, 1991) and is based on both biological and clinical rationales. Removal of the primary tumour may increase the rate of growth of micrometastases, explained by the presence of a circulating growth factor and prevented by administering chemotherapy prior to surgical excision (Fisher *et al*, 1983). More recent work has focused on the role of angiostatin, a circulating inhibitor of angiogenesis produced by tumour cells. Following primary tumour removal, levels of angiostatin fall and rapid growth of distant metastases ensues (O' Reilly *et al*, 1994). Such rapid growth of metastases is inhibited by administration of recombinant angiostatin (O' Reilly *et al*, 1994). Another group has proposed that, as the number of tumour cells increase, so does the likelihood of development of chemoresistant clones (Goldie & Coldman, 1979). According to the hypothesis, development of chemoresistant clones may be minimized by early administration of chemotherapy.

Potential clinical advantages are that early treatment of micrometastases may avoid their rapid growth following tumour removal and prevent emergence of resistant clones. Pre-operative reduction in tumour volume may render inoperable tumours operable, or allow breast conservation when mastectomy had been the only surgical option prior to systemic treatment. In addition, neoadjuvant therapy allows an objective assessment of response and allows study of the relationship between pathological and molecular markers and responsiveness. Most studies of neoadjuvant therapy in breast cancer have focused on the role of chemotherapy and are worthy of comment to illustrate the role of such treatment.

Neoadjuvant or primary systemic therapy was initially developed in the 1970s for the management of locally advanced breast cancer when aims of treatment were to prevent disease progression, with the emphasis on local control rather than on management of systemic disease. Early studies, however, suggested that, when compared to local treatment alone, survival was prolonged (Rubens *et al*, 1980; Valagussa *et al*, 1990). In addition, administration of chemotherapy prior to local treatment caused a reduction in the size of primary tumour, thereby facilitating surgery and/or radiotherapy (Forrest *et al*, 1991; Valagussa *et al*, 1990).

The advantages of primary chemotherapy in managing locally advanced breast cancer are, therefore, that it reduces tumour burden leading to improved local control, it checks postoperative growth in metastases and allows evaluation of its efficacy, which will than improve selection of subsequent adjuvant therapy (Trimble *et al*, 1993). Possible disadvantages of this approach are that, if ineffective, it delays definitive treatment and thus may compromise local control. Wound healing may be affected due to reduced tumour vascularity occurring as a consequence of treatment (Trimble *et al*, 1993).

The potential role of tamoxifen as primary treatment of breast cancer was first proposed in 1982 (Preece *et al*, 1982). The use of tamoxifen as sole treatment for locally advanced disease has been explored in two studies: in one study treatment with tamoxifen and radiotherapy were compared, with similar survival rates in the two groups (Robertson *et al*, 1994). In the other study tamoxifen and CMF chemotherapy were compared in 181 patients over 65 years with recurrent breast cancer and it was found that survival rates tended to favour tamoxifen, with comparable response rates (Taylor, 1986).

The majority of studies aimed at determining the role of tamoxifen in the management of operable breast cancer have focused on its use as primary treatment in elderly patients, in whom minimal treatment was considered advantageous (Allan *et al*, 1985; Helleberg *et al*, 1982). Such studies reported response rates to primary treatment with tamoxifen ranging from 40-70% (Allan *et al*, 1985; Anderson *et al*, 1991; Horobin *et al*, 1991; McDonald *et al*, 1990). However, in most studies of tamoxifen alone or in comparison with surgery, unacceptably high rates of tumour regrowth occurred (Bergman *et al*, 1995; Horobin *et al*, 1991; Robertson *et al*, 1992). In one study the use of tamoxifen alone was compared with surgery and adjuvant tamoxifen and a small, but significant survival advantage in the group who underwent surgery was found (Bates *et al*, 1991). A more recent study investigating the role of oestrogen receptor (ER) expression in determining response to primary tamoxifen found that a significantly higher proportion of ER-positive tumours responded than ER-negative. Fewer patients developed tumour regrowth in the ER-positive group, particularly in those patients who responded to tamoxifen in the first six months of treatment (Willsher *et al*, 1997). Treatment with tamoxifen alone in elderly women is only acceptable in those who are very frail and have strongly ER-positive tumours (Dixon, 1992).

The importance of ER expression was further highlighted in a study of women of all ages with operable breast cancer treated with neoadjuvant systemic therapy (Anderson *et al*,

1991). At the commencement of the study all patients were treated with primary tamoxifen, but initial results demonstrated that ER-poor tumours did not respond to tamoxifen and such patients were subsequently treated with primary chemotherapy (Anderson *et al*, 1991). Long-term follow-up in these patients show similar survival rates in the tamoxifen- and chemotherapy-treated groups (Cameron *et al*, 1997a).

Assessing tumour response to neoadjuvant therapy:

In order to determine the efficacy of neoadjuvant treatment it is necessary to define its purpose. For patients with large primary breast tumours, tumour regression is an endpoint in itself, and is used to assess response to therapy. Potential methods of assessment are by clinical, mammographic and ultrasound measurements of tumour volume. Clinical measurements are prone to error: the method is indirect, influenced by oedema and obesity (Dixon *et al*, 1984) and are observer-dependent (Warr *et al*, 1984). Mammography is well established as an objective method of assessing breast tumours, but the frequency with which it can be performed is limited by radiation dose. In lobular carcinoma, tumour margins are poorly defined on mammography (Hilleren *et al*, 1991) and this precludes estimation of small variations in size. Ultrasonography is simple to perform, provides a permanent record of tumour size and may be carried out frequently. It provides an alternative method of assessment of response to primary systemic therapy. In a study comparing clinical, mammographic and ultrasonographic tumour measurements and correlating them with pathological size, the best results were obtained using ultrasound which was applicable to a larger group of patients than mammography (Forouhi *et al*, 1994). Clinical size was less closely correlated with pathological size. Thus serial ultrasound assessments of tumour volume provide a practical and accurate method of monitoring tumour response (Forouhi *et al*, 1994).

Definition of response was defined by UICC based on clinical measurements. Response was defined as a fall of at least 50% in the product of two orthogonal diameters of a tumour for at least one month (Hayward *et al*, 1977). This method of assessing response is clearly of limited value in patients treated for three months. An alternative method is to define the change in tumour volume by ultrasound. Observer variation is less than 10% (Forouhi *et al*, 1994), so that changes in volume of greater than 10% are significant. For the purposes of this thesis, a reduction in tumour volume of 25% or more was classified as a response. Tumour volume was calculated according to the equation:

$$\text{Tumour volume} = A \times B \times C \times \pi / 6$$

where A is the transverse diameter

B is the anteroposterior diameter

C is the depth as assessed on ultrasound.

Resistance to tamoxifen treatment:

As has been stated, approximately one-third of all breast cancers respond to tamoxifen, the majority, if not all, of these are oestrogen receptor positive. Response rates of ER-positive tumours rarely exceed 70% (Allan *et al*, 1985) and the majority of tumours which do initially respond to tamoxifen subsequently develop resistance, which may occur following weeks or years of treatment (Lykkesfeldt, 1996). Between 35 and 60% of those with an initial response to tamoxifen will respond to second line endocrine therapy, indicating that development of tamoxifen resistance is not an indicator of complete loss of hormone responsiveness. Understanding mechanisms of tamoxifen resistance is of value in that it may identify accurate predictors of response and allow development of drugs that do not lead to such resistance. With increasing use of tamoxifen in the neoadjuvant setting as outlined above, tumour responsiveness can be assessed and patients with non-responding tumours offered alternative therapy.

Resistance to tamoxifen may be primary or acquired. The two major reasons for primary resistance are that the tumour may be inherently autonomous, or that intra-tumour tamoxifen levels are insufficient to compete effectively with endogenous oestrogens (Miller, 1996a). Other mechanisms may be involved in primary resistance and these will be addressed when considering acquired resistance, which will provide the main focus of the discussion. Since the main effect of tamoxifen is mediated via the oestrogen receptor (ER) signalling pathway, change in any step of this pathway may render tumour cells resistant. There may be an alteration in uptake or metabolism of tamoxifen into the cell, loss or decrease in ER expression, expression of variant or mutant ER forms, intact ER but loss of co-activators, modifications of oestrogen response elements (EREs) or altered post-receptor events. Non-ER related changes may also contribute to tamoxifen resistance (Lykkesfeldt, 1996).

Tamoxifen may exert oestrogen agonistic activity, depending on the organ involved, such as the endometrium, and variations in local intracellular tamoxifen concentrations (Jordan & Murphy, 1990). Agonist activity may be observed when tamoxifen is present at low intracellular concentrations (Osborne *et al*, 1991; Osborne *et al*, 1992). Such reduced intracellular levels following an initial period of successful treatment with tamoxifen may be due to decreased uptake into or increased efflux of tamoxifen from cells. P-glycoprotein is involved in efflux of chemotherapeutic agents from cell and elevation in its expression has been found in non-responders to tamoxifen treatment (Keen *et al*, 1994).

About 20-40% of patients who initially present with ER-positive tumours are found to have ER-negative tumours at the time of recurrence following adjuvant tamoxifen (Johnston *et al*, 1995). In the majority of cases of resistance, however, ER-positivity is maintained, indicating that resistance is unlikely to have developed as a result of an outgrowth of ER-negative clones following response of hormone-dependent clones to tamoxifen (Miller, 1996a).

Expression of mutant or variant ER in tamoxifen-resistant breast cancers is a rare phenomenon and cannot account for most oestrogen-independent, tamoxifen-resistant breast tumours (Karnik *et al*, 1994). In one study, 18 of 20 tamoxifen-resistant tumours did not have mutations in any of 8 exons of ER complementary DNA (Karnik *et al*, 1994). Despite this, several mutations have been identified in breast tumours, including the exon 5 deletion splice variant of ER (Daffada *et al*, 1995), which may be translated into a protein capable of transcription without ligand binding (McGuire *et al*, 1991). Another ER mutant which has been described follows a point mutation in the ER region upon which activity of the AF-2 transcriptional activation domain depends, leading to strong agonist activity of tamoxifen (Mahfoudi *et al*, 1995). Relevance of such findings depends on variant ER protein being translated from RNA and there is little evidence for this (Madsen *et al*, 1995).

The function of the ER depends on the presence of several co-activators, which associate with the activated conformation of the ER. Changes or loss of these may render breast cancer cells resistant to antiestrogens (Lykkesfeldt, 1996). Once ER-bound, ligand-dependent phosphorylation is important for activating transcription. This phosphorylation may occur via ligand-independent pathways, with cross-talk between a growth factor signalling pathway and the ER, rendering tamoxifen ineffective. Alteration in levels of cyclic AMP or an increased number of protein kinase A catalytic subunits cause cells to respond to tamoxifen as an oestrogen agonist rather than antagonist (Fujimoto & Katzenellenbogen, 1994). Modification of the oestrogen response elements (ERE), to which the activated ER dimer binds, may render it more sensitive to agonist activity of tamoxifen (Dana *et al*, 1994).

Other potential mechanisms of resistance are not mediated by the oestrogen receptor and include constitutive expression of growth factors such as transforming growth factor-alpha (Nicholson *et al*, 1994b), upregulation of growth factor receptors, e.g. epidermal growth factor receptor (Nicholson *et al*, 1994a) and c-erbB-2 (Borg *et al*, 1994).

This overview of potential mechanisms of tamoxifen resistance demonstrates the complexity of its action and the inability of ER expression alone to identify likely responders. More accurate predictors of initial response to treatment and likelihood of development of resistance would be of benefit.

Prediction of tamoxifen sensitivity:

Potential mechanisms of resistance, discussed in the previous section, demonstrated the complexity of interaction between tamoxifen and the oestrogen receptor, with involvment of other factors. Whilst ER is an important predictor of tamoxifen sensitivity, other more accurate predictors of response would be of benefit in the clinical setting.

Hormone receptors:

The initial step in oestrogen action is the binding of oestrogen to its receptors. Thus determination of the presence or absence of such receptors in the breast may provide useful information on endocrine sensitivity. Between sixty and seventy five percent of breast cancers are ER-positive; within this group there is a continuous range of values. The level of oestrogen receptor is affected by age, menopausal status and tumour grade. Expression increases with increasing age (Clark *et al*, 1984), is more common in postmenopausal women and in lower grade tumours (Thorpe & Rose, 1986). Interestingly, the proportion of ER-positive tumours is increasing; whether this is due to increasingly sensitive assays or a change in the nature of tumours is unclear (Pujol *et al*, 1994).

A highly significant correlation has been found between response to neoadjuvant tamoxifen and ER expression based on immunocytochemical techniques on fine needle aspiration cytology (Anderson *et al*, 1989; Coombes *et al*, 1987; Osborne *et al*, 1980). Evaluation of ER content has become standard practice in determining suitability of patients for primary endocrine treatment. Despite this selectivity, response rates rarely exceed 70% (Allan *et al*, 1985; Osborne *et al*, 1980). Lack of ER-expression predicts for tamoxifen insensitivity (Anderson *et al*, 1989; Robertson *et al*, 1994) and this has been demonstrated in a recent overview of adjuvant tamoxifen (Early Breast Cancer Trialists' Collaborative Group, 1998). Previous suggestions that up to 10% of ER-negative tumours respond are now thought to be related methodological errors of ER assessment. Addition of progesterone receptor (PgR) assessment provides greater accuracy in predicting response, those tumours expressing both receptors have response rates of 66-83% (Osborne *et al*, 1980). However, ER and PgR positivity as a pre-requisite for primary endocrine therapy would result in failure to respond in approximately 20% of patients and would exclude ER-positive, PgR-negative tumours that may respond (Osborne *et al*, 1980).

Proliferation:

Following binding of oestrogen to its receptor, it exerts its action at the cellular level by stimulating proliferation. An alternative strategy in determining sensitivity to endocrine therapy would be to assess changes in proliferation. Ina study of primary breast cancers a reduction in proliferation was detected using Ki-S1 antibody following treatment with tamoxifen (Keen *et al*, 1997). In a more recent study, an early reduction in proliferation was observed using the Ki-67 antibody during treatment with tamoxifen (Makris *et al*, 1998). Many potential surrogate markers of proliferation of breast cancers have been studied. The classic measure in solid tumours that have been fixed and processed is the mitotic index. Indeed the total number of mitotic figures in 10-high power fields is one of the three cardinal features used to assess grade of a clinical breast cancer (Bloom & Richardson, 1957). An alternative of assessing mitotic figures is the use of immunohistochemistry to identify cells that express antigens associated with proliferation. A number of such antigens are known, including Ki-67, PCNA and Ki-S1. There are data to suggest that PCNA and Ki-S1 staining may vary with different concentrations of antibody whereas MIB-1 antibody for Ki-67 is more robust and not expressed in non-proliferating cells (McCormick *et al*, 1993). Thus in the present study, changes in proliferation were assessed with MIB-1 antibody to Ki-67 to determine whether such a marker predicts sensitivity to tamoxifen.

Other predictors of response have been studied. Bcl-2 is a protein involved in inhibition of apoptosis and may be important in modulation of hormone responsiveness (Miyashita & Reed, 1992). Bcl-2 expression has been found to be a highly significant predictor of endocrine responsiveness (Gee *et al*, 1994). Another marker which has found significance in predicting endocrine responsiveness is pS2 protein (Soubeyran *et al*, 1996) Response rates in tumours expressing both pS2 and ER were 60%, in those expressing one factor alone were 45% and in tumours expressing neither pS2 or ER were 8% (Soubeyran *et al*, 1996). In one study, pS2 was found to be a more accurate predictor of response than ER (Soubeyran *et al*, 1996).

Angiogenesis:

In this section the process of angiogenesis is outlined and mechanisms by which a tumour becomes angiogenic considered. The relationship between oestrogen and angiogenesis is explored and the clinical applications of the study of angiogenesis are reviewed. Specific consideration is given to the potential role of angiogenesis as a predictor of sensitivity to primary endocrine therapy, which provides the basis of this thesis.

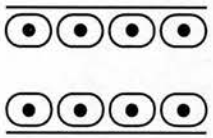
Tumour development comprises unregulated growth of the abnormal cell and changes in the surrounding microenvironment. Thus solid tumours comprise two components: tumour cells and stromal cells within the surrounding extracellular matrix (Dvorak, 1986). There is growing experimental evidence demonstrating that stromal and inflammatory cells such as endothelial cells, fibroblasts and macrophages play an active role in tumour development. Evidence has been accumulating since the early 1970s that new vessel formation or angiogenesis is a pre-requisite for tumour growth beyond 2-3mm^3 and for metastasis (Folkman, 1990; Folkman, 1994). This evidence is most clearly illustrated clinically in malignant melanoma and can be observed from an early stage. Prior to the onset of angiogenesis, tumours are thin and slow growing, but with the introduction of new vessels rapid tumour growth and progression take place (Srivastava *et al*, 1988).

Physiological Angiogenesis:

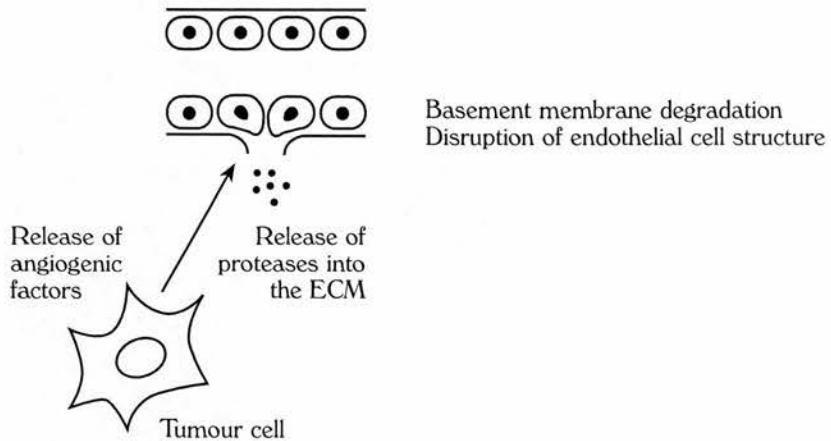
Angiogenesis plays an essential physiological role in embryogenesis and foetal development and in the female reproductive cycle. In the normal adult male, angiogenesis is usually quiescent, but is stimulated during wound healing. As well as being essential for tumour progression, angiogenesis is involved in certain benign disease states including the proliferative retinopathies (Gole *et al*, 1990) and rheumatoid arthritis (Colville- Nash & Scott, 1992; Peacock *et al*, 1992).

Angiogenesis is a multistep process involving alterations in endothelial cells and extracellular matrix remodelling. The process is summarised diagrammatically on the following page. There is local degradation of basement membrane caused by release of proteases in the extracellular matrix, and endothelial cells around the site of disruption change shape and invade stroma. These endothelial cells are stimulated to proliferate at the leading edge of the migrating column, adhering to each other tightly to form tubes, which coalesce to form loops. At this point blood starts to circulate in newly formed vessels.

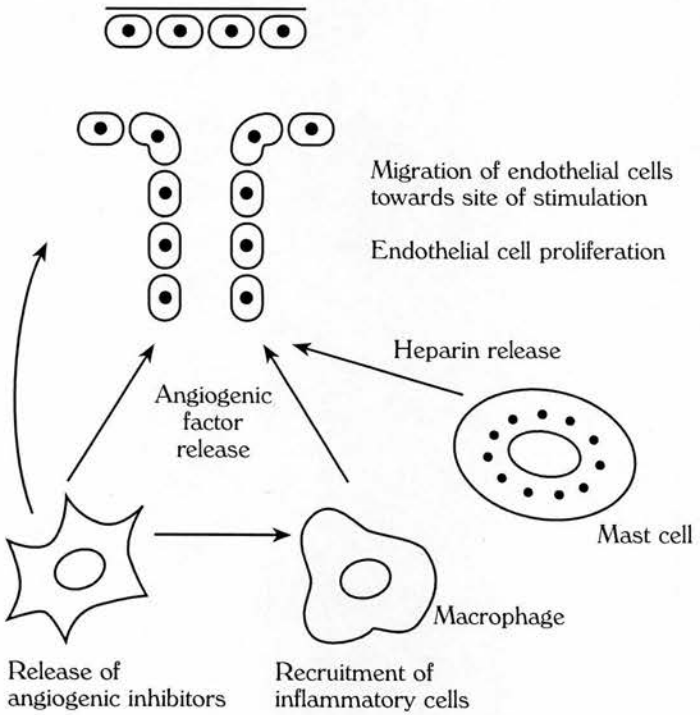
1. Quiescent endothelium



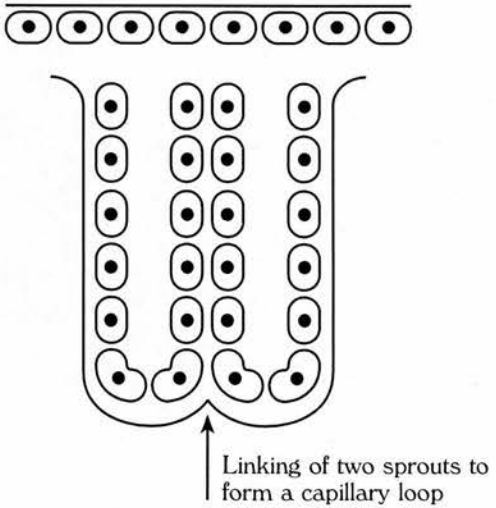
2. Tumour cells act as angiogenic stimuli



3. Endothelial cells are stimulated to migrate and proliferate by released angiogenic factors



4. Following lumen development two capillary sprouts coalesce to form a loop - blood flow commences



Tumour angiogenesis:

This complex angiogenic process involves multiple paracrine and autocrine interactions of tumour and stromal components (Casey *et al*, 1995; Weidner *et al*, 1992), and the mechanisms of control involve both stimulatory and inhibitory factors (Hanahan & Folkman, 1996). During tumour development, new vessel formation occurs as an early event, which precedes tumour invasion and is a discrete component of the tumour phenotype, rather than resulting from tumour hypoxia as the growing mass outgrows its blood supply. This is illustrated in experiments based on transgenic mouse models, in which a subset of tumour cells appeared to stimulate angiogenesis in the neighbouring stroma before tumour invasion occurred (Hanahan & Folkman, 1996). This concept is supported by the observation that angiogenesis occurs during the pre-invasive stages of breast and cervical carcinomas: DCIS (Guidi *et al*, 1994; Weidner *et al*, 1992) and CIN II-III respectively (Guidi *et al*, 1995). Thus, the switch to an angiogenic phenotype is a well-defined step occurring early in tumour development.

Angiogenic Inducers:

Experimental evidence suggests that angiogenesis is stimulated by release of soluble factors produced by tumour cells or surrounding stromal and inflammatory cells. Several angiogenic factors have been identified: two of current interest because of their potential for therapeutic use will be described here, the fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF):

Acidic and basic fibroblast growth factors (FGF-1 and FGF-2).

FGF-1 and 2, known as acidic and basic fibroblast growth factors, are members of the family of fibroblast growth factors, of which nine are currently identified. Family members have varying degrees of structural homology and are produced by a variety of cells (Christofori, 1997). They are involved in many physiological and pathological processes, with a wide range of biological activities and target cells, making determination of precise mechanisms and the roles they play in individual processes difficult. FGF-1 is found in high levels in neural tissue (Basilico & Moscatelli, 1992) and FGF-2 is more widely distributed, it has been isolated from brain, kidney, placenta and tumour cells (Christofori, 1997). Both FGF-1 and 2 function through autocrine and paracrine mechanisms, and play an important role in embryonic development and wound healing. They are mitogenic for endothelial cells (Christofori, 1997) and induce the endothelial cell production of proteases, which degrade basement membrane.

FGF-1 and 2 are expressed in a range of tumours (Relf *et al*, 1997), including breast carcinoma, although their precise contribution to tumour angiogenesis is difficult to assess because of the pleiotrophic nature of their actions. In order to exert their angiogenic action, the FGFs must be released into the extracellular matrix (ECM) and bind to their endothelial cells receptors. However, they lack the signal sequence for secretion (Mignatti & Rifkin, 1991) and the mechanism of their transport into the ECM is not clearly understood. Two possible mechanisms of release have been proposed: one involves their release following cell damage, the second implicates an alternate secretory pathway involving translocation of molecules. Recently it has been suggested that ECM release of FGF-2 may involve its displacement from heparin, to which it is bound, by a secreted FGF-binding protein (FGF-BP). This may be an important component of the angiogenic switch in some cancers (Czubayko, 1997).

Vascular endothelial growth factor (VEGF).

VEGF is expressed on a variety of normal cells and is secreted by a number of human tumour cell lines (Senger *et al*, 1986). It is of particular interest because it acts specifically on endothelial cells (Ferrara & Henzel, 1989), binding exclusively to these target cells which express one of the two endothelial cell specific tyrosine kinase receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2) (Jakeman *et al*, 1992), acting as a paracrine mediator. Production of VEGF can be induced by a variety of cytokines such as platelet derived growth factor (PDGF) (Colville-Nash & Willoughby, 1997) and transforming growth factor β (TGF-β) (Pertovaara *et al*, 1994), and it works synergistically with FGF-2 (Pepper *et al*, 1992). In addition, VEGF is induced by hypoxia, through upregulation of the KDR receptor, suggesting a role in the maintenance of the normal cellular environment (Dvorak *et al*, 1995). It is structurally homologous to vascular permeability factor (VPF), which is known to cause protein extravasation (Dvorak *et al*, 1995). This process is recognized as an important early step in angiogenesis, releasing fibrin in the extracellular matrix (ECM) (Senger, 1996). Fibrin encourages new vessel growth and is structurally important for changing the ECM.

VEGF occurs as four isoforms generated by the alternative splicing of RNA, two secreted and two cell-associated; the secreted forms are heparin bound, like the FGFs. The binding of heparin is important in reducing proteolytic cleavage, providing an extracellular store of growth factors that can be rapidly mobilised and facilitating presentation of the growth factors to the endothelial cell (Vlodavsky *et al*, 1990).

Increased VEGF expression in tumours has led to study of its potential role as a prognostic indicator in breast cancer. Several studies have found an association between tumour VEGF expression and relapse free or overall survival, with significant shorter survival times in those patients with VEGF-rich tumours. (Gasparini *et al*, 1997; Toi *et al*, 1994). Changes in serum levels of VEGF correlated with response in those patients with metastatic renal carcinoma treated with interferon α (Vermeulen *et al*, 1997a).

The role of VEGF in certain benign disorders, in which angiogenesis is known to be important, such as diabetic proliferative retinopathy (Aiello *et al*, 1994) and rheumatoid arthritis (Fava *et al*, 1994) has led to development of potential therapeutic strategies adopting the use of VEGF and its antibody. VEGF may play a role in the treatment of limb and coronary ischaemia, with injection of recombinant VEGF resulting in improved collateral

circulation in pigs (Pearlman *et al*, 1995; Takeshita, 1994). Development of an anti-VEGF antibody may provide an exciting anti-angiogenic drug for the treatment of solid tumours in which VEGF is overexpressed, including breast cancer (Jin Kim *et al*, 1993). It is likely to be of most use in combination with other therapeutic strategies as VEGF is only one of several growth factors involved in the process of tumour angiogenesis. Nevertheless results of the current Phase I studies of this antibody are awaited with interest.

The increased expression of multiple growth factors, including FGF-1, FGF-2 and VEGF, in breast cancer cells demonstrates the complexity of the angiogenic response and the difficulty that therefore arises in attempting to develop anti-angiogenic therapeutic strategies. The binding of such growth factors to heparin provides a potential common mechanism for their inhibition and suramin analogues have been developed and are currently in Phase I clinical trials (Harris *et al*, 1996).

Angiogenic inhibitors:

Thrombospondin:

Thrombospondin (TSP) is a matrix-bound glycoprotein which plays a role in tumour cell proliferation, differentiation and angiogenesis (Bornstein, 1992). It is an endogenous inhibitor of angiogenesis counteracting the action of positive stimulators, and must be overcome in order for tumour angiogenesis to take place. High levels are produced by fibroblasts and by some epithelial cells but its production is decreased in malignancy. TSP is regulated by wild type p53 tumour suppressor gene, and inactivation of this gene leads to decreased levels of thrombospondin (Rastinejad *et al*, 1989). Patients with Li Fraumeni syndrome, characterized by development of malignancies of breast, brain and connective tissue, have only one copy of the p53 tumour suppressor gene and, when this allele is deleted, TSP production decreases and angiogenesis is switched on (Bouck, 1996). The therapeutic potential of TSP in breast cancer is illustrated by an in vivo study in which human breast cancer MDA-MB-435 cells were transfected with complementary DNA encoding TSP-1, resulting in a reduction in vessel density and metastasis (Weinstat-Saslow *et al*., 1994).

Angiostatin:

Angiostatin is another potent inhibitor of angiogenesis, produced in response to the growth of a solid tumour and suppressing the growth of distant metastases (O' Reilly *et al*, 1994). Angiostatin was identified using the Lewis lung carcinoma model (O' Reilly *et al*, 1994), in

which removal of the primary tumour stimulated growth of lung metastases. Histologically, there was intense neovascularization around the metastases. In animals in whom the primary tumour remained, no such neovascularization was seen and metastases remained small. These effects appeared to be mediated via a circulating inhibitor of angiogenesis, angiostatin, which is a fragment of the large protein, plasminogen. Plasminogen itself has no anti-angiogenic activity (O' Reilly *et al*, 1994). The existence of angiostatic fragments of larger protein molecules which do not themselves have any anti-angiogenic activity may indicate a role of these molecules in maintaining the normal quiescent state of angiogenesis in adults whilst allowing temporary stimulation of angiogenesis during wound healing. In tumour formation, it is not clear whether tumour cells produce angiostatin or a protease which cleaves plasminogen to produce angiostatin (O' Reilly *et al*, 1994).

Endostatin:

Endostatin is a protein fragment of collagen XVIII and acts in a similar fashion to angiostatin. It specifically inhibits endothelial cell proliferation and recombinant endostatin inhibits angiogenesis, growth of primary tumours and metastases. Like angiostatin, it acts by increasing the apoptotic rate, with little effect on proliferation (O' Reilly *et al*, 1997).

The identification of circulating stimulators and inhibitors of angiogenesis has led to the theory that there is a switch from the non-angiogenic to the angiogenic phenotype which occurs when the balance of concentrations of angiogenic stimulators outweighs the inhibitors (Hanahan & Folkman, 1996). This then allows tumour growth beyond 2-3mm^3 and facilitates metastasis. The potential benefits of the discovery of such angiogenic and angiostatic molecules for treatment of malignancies are evident; production of antibodies to inducers and of inhibitory factors may lead to exciting anti-angiogenic strategies, and such work is ongoing.

Hormonal Control of Angiogenesis.

The aetiological role played by oestrogen in the development of breast cancer is well recognised, prolonged exposure to oestrogen resulting in an increased risk of developing breast cancer of 5% per year (Abeloff *et al*, 1995). Several manifestations of vascular disease suggest that endothelial cell activity may also be regulated by hormones, and this has led to interest in the potential link between hormonal control of tumour growth and tumour angiogenesis. Thus, systemic lupus erythematosus (SLE) and Takayasu's arteritis, both characterized by endothelial cell proliferation, commonly affect women of childbearing age. SLE is exacerbated by pregnancy and by use of the oral contraceptive pill (Ahmed *et al*, 1985). Additionally, cutaneous haemangiomas are more commonly found in female children (Ezekowitz *et al*, 1992), spider naevi characteristic of liver disease are related to high levels of circulating oestrogens (Sarkany. & Graham-Brown, 1991) and oestrogens protect against atherosclerosis (Matthews *et al*, 1989; Williams *et al*, 1990).

Experimental evidence supports the role of hormones, particularly oestrogens, in angiogenesis. For example, oestrogen added to human umbilical vein endothelial cells (HUVECs) grown in culture in oestrogen-free medium increased endothelial cell proliferation, cell attachment to laminin and enhanced the ability of HUVECs to organize into tubular networks, all of which are important components of the angiogenesis process (Morales *et al*, 1995). Another experiment in which cultures of HUVECs were exposed sequentially to tumour necrosis factor-α (TNF-α) and then to oestrogen showed that apoptosis caused by TNF-α was reversed by oestrogen. This suggests that oestrogen may play a role in the preservation of endothelial cells by inhibiting apoptosis (Sypridopoulos *et al*, 1997). This protection is probably mediated through the oestrogen receptor since a specific receptor antagonist abrogated the effect.

Other studies illustrate the angiostatic effect of anti-oestrogens, which are of particular interest in this thesis. Gagliardi and Collins showed that the partial oestrogen antagonists, clomiphene, tamoxifen and nafoxidene, and the pure oestrogen antagonists, ICI 164, 384 and ICI 182, 780, inhibited angiogenesis in the chick egg chorioallantoic membrane (Gagliardi & Collins, 1993). As the effect was maintained despite exposure to high levels of oestrogen it was unlikely to be mediated via the oestrogen receptor. Follow up work showed that porcine pulmonary artery endothelial cells, whose growth was stimulated by the angiogenic peptides, bFGF and VEGF, could be inhibited by tamoxifen in a dose-dependent manner (Gagliardi *et*

al, 1995). Inhibition was most impressive in tumours stimulated by both bFGF and VEGF and was associated with changes in endothelial cells suggestive of apoptosis (Gagliardi *et al*, 1995). Results from this study suggest that the anti-angiogenic effect of anti-oestrogens is mediated through direct inhibition of growth factor stimulated endothelial cell growth (Gagliardi *et al*, 1995).

Tamoxifen also inhibits angiogenesis in the MCF-7 breast cancer xenografts, which are oestrogen dependent (Lindner & Borden, 1997). Ten weeks of tamoxifen treatment of nude mice bearing these tumours resulted in a 68% decrease in the number of vessels at the tumour periphery and growth inhibition by 85% compared with the control group (Lindner & Borden, 1997). Similar work in our laboratory compared the effect of tamoxifen (2.5mg over 60 days) following oestrogen stimulation on the oestrogen receptor-positive breast cancer cell line, ZR-75 and -negative cell line, MDA-MB-231. An early reduction in microvessel density was observed in ZR-75 but not MDA-MB-231 tumours (Cameron *et al*, 1996). Preliminary work on primary oestrogen receptor positive breast cancers treated with three months' tamoxifen prior to surgery showed a reduction in microvessel density in responding tumours, compared to no change or an increase in microvessel density in those which did not respond (Marson *et al*, 1998). It is not clear whether such changes occurred as a primary event or as a consequence of tumour regression.

Mechanisms by which oestrogens might control angiogenesis have also been investigated. Whilst some actions appear to be mediated through the oestrogen receptor (Sypridopoulos *et al*, 1997), this is not always how they achieve their effect (Gagliardi & Collins, 1993). Oestrogen receptor expression by endothelial cells has been demonstrated in cell culture (Gagliardi & Collins, 1993), but not in primary tumours. An alternative potential mechanism of action is by enhancement of the action of angiogenic peptides. VEGF is produced in oestrogen-rich cells and the anatomical and temporal pattern of its expression in the uterus and ovaries suggest hormonal regulation of its expression (Shweiki *et al*, 1993). Male rats treated with tamoxifen or placebo injected with VEGF-impregnated matrigel showed a reduction in gel microvessel densities in the tamoxifen treated group compared with controls, suggesting that tamoxifen affects VEGF-mediated angiogenesis (McNamara *et al*, 1998). Angiogenesis was decreased in ovariectomised mice despite the presence of bFGF, suggesting that the angiogenic effects of bFGF may be reduced in the absence of oestradiol (Morales *et al*, 1995). Tamoxifen enhanced secretion of transforming growth factor-β (TGF-

β) in ER positive and negative breast cancer cell lines (Arteaga *et al*, 1988) and TGF-β inhibited endothelial cell proliferation induced by bFGF and VEGF (Arteaga *et al*, 1988).

The potential role of angiogenic factors in the evolution of oestrogen-independent breast cancers from those which were initially oestrogen dependent has been investigated. Resistance to tamoxifen presents a major problem in the management of breast cancer, both in the adjuvant setting and in those patients with advanced disease as previously discussed. The MCF-7 breast cancer cell line forms slow-growing, poorly-vascularised tumours which are oestrogen dependent and tamoxifen sensitive when implanted into nude mice. However, transfection of fibroblast growth factor 4 (FGF-4) into MCF-7 cells produced lines that are highly tumorigenic and metastatise in ovariectomised or tamoxifen treated nude mice, with these tumours being paradoxically stimulated by tamoxifen and inhibited by oestrogen (McLeskey *et al*, 1993; Kurebayashi *et al*, 1993). In contrast, transfection of the same cell line with VEGF resulted in rapidly growing, highly vascular tumours which maintained oestrogen dependence and tamoxifen sensitivity, with no evidence of metastasis (Zhang *et al*, 1995).

These cell lines may be used to mimic progression from oestrogen dependent to oestrogen independent breast cancer. For example, in one study, MCF-7 cells transfected with VEGF were mixed with the ER negative cell line MDA-435S and tumour growth in both cell lines was stimulated by the presence of VEGF (Macaulay *et al*, 1995). It seems that production of angiogenic factors by the ER positive subpopulation of cells influenced ER negative cells. This is likely to mimic breast cancers that are recognized to show marked heterogeneity. The addition of tamoxifen in this group inhibited growth stimulation (Macaulay *et al*, 1995).

Clinical Applications of Angiogenesis:

Effect of Surgery on Angiogenesis.

There is evidence that the healing wound is a permissive site for tumour metastasis (Murthy & Goldschmidt, 1989). Tumour cells reaching a colorectal anastomosis or laparotomy wound within two hours post-operatively have a thousand-fold greater risk of metastasis compared to normal tissue (Skipper *et al*, 1989). The timing of development of metastases following breast cancer surgery has two peaks. The first peak occurs during the first three post-operative years and may be due to the release of angiogenic cytokines during wound healing; the second is a number of years later, indicating a variable rate of metastasis growth (Baum & Badwe, 1994). The angiogenic inhibitor, angiostatin, is produced by tumour cells and is absent following excision of the primary tumour, leading to rapid development of lung metastases (O' Reilly *et al*, 1994). The concept that a tumour produces an inhibitor of angiogenesis has implications for the surgical management of cancer patients. Surgery may also facilitate metastasis by shedding of tumour cells into the circulation. In the immediate post-operative period, breast cancers with high vascular density shed more tumour cells into the circulation than those with low density (McCulloch *et al*, 1995).

Angiogenesis as a Prognostic Indicator.

Weidner and colleagues were the first to suggest that microvessel density might provide prognostic information in breast cancer. In a series of forty-nine patients a significant correlation was found between the presence of metastasis and microvessel density performed following immunohistochemical staining of endothelial cells with Factor VIII antibody (Weidner *et al*, 1991). Since then, numerous studies have been performed, the majority of which have confirmed these findings and have found correlation between microvessel density and relapse-free and overall survival (Bosari *et al*, 1992; Horak *et al*, 1992). In a study of lymph node-negative breast cancer patients, microvessel density was found to be an independent prognostic indicator for relapse free survival (Gasparini *et al*, 1994). Whilst some studies have not found microvessel density to be a useful prognostic indicator, these discrepancies are likely to be due to methodological variation (Hall *et al*, 1992; Van Hoef *et al*, 1993). In this respect, there is debate concerning assessment of tumour vascularity by microvessel counts, focusing on two main issues: the antibody used to stain endothelial cells and the method of counting.

Pan-endothelial cell markers stain all endothelial cells and those most commonly used are antibodies to Factor VIII, CD31 and CD34. Antibodies that are specific for activated or proliferating cells are under development. The FVIII antigen plays a role in platelet aggregation and adhesion (Fajardo, 1989), and its antibody is a commonly used endothelial cell marker (Burgdorf *et al*, 1981). Antibodies against FVIII consistently stain endothelial cells and give more reproducible staining than other markers. They stain large vessels but are decreased or absent in small microvessels, and also stain lymphatics. CD31 plays a role in platelet adhesion during inflammatory processes and wound healing (Parums *et al*, 1990). Antibody to CD31 was recently recommended in guidelines for a proposed standard method of microvessel counting (Vermeulen *et al*, 1996). It is a sensitive, pan-specific marker of endothelial cells, which gives consistently higher counts than the FVIII antibody (Horak *et al*, 1992; Martin *et al*, 1997). Unlike FVIII antibody, it does not stain lymphatics, but does stain some inflammatory cells. However, it is associated with more frequent antigen loss following tumour fixation, and staining tends to be weaker than that obtained by FVIII antibody (Martin *et al*, 1997). CD34 is a cell adhesion molecule that plays a role in leucocyte adhesion and endothelial cell migration (Schlingemann *et al*, 1990). In a recent study it was found to be the most strongly expressed antigen, staining more vessels reproducibly than CD31 and FVIII (Martin *et al*, 1997).

One of the criticisms levelled at the technique of microvessel counting is that counts include all vessels and are not specific for new or angiogenic vessels. The importance of this is uncertain, as angiogenic vessels are often leaky and ineffective in transporting blood and so are unlikely to affect tumour growth in its early stages. However, attempts have been made to develop antibodies that are specific to activated/ proliferating endothelial cells. One such antibody is E-9 and in a comparative study with CD31 was found to stain vessels in or around the tumour with a consistently greater intensity than CD31 (Wang *et al*, 1994). More recently the antibody to CD105, which preferentially reacts with endothelial cells in angiogenic tissues, was compared with CD34 antibody in a series of breast tumours and was found to correlate with tumour prognosis more accurately than CD34 (Kumar *et al*, 1999). Further work needs to be carried out on this and other potentially specific markers to determine their value in clinical practice.

Quantifying angiogenesis requires consideration of several variables, the most notable of which is choice of counting area. Counts are often performed in the areas of highest

vascularity or 'vascular hot spots' (Weidner *et al*, 1991). The rationale behind this is that, whilst not all tumour cells are angiogenic, it is the highly angiogenic tumour cell clones that are most likely to behave aggressively and to metastasise. These areas are most commonly found on the tumour periphery close to viable tumour, but tumour heterogeneity in breast carcinomas provides particular problems.

To reduce inter-observer variability it is important to define microvessels. The generally accepted definition is any single stained endothelial cell or cell cluster which is clearly separate from adjacent microvessels, tumour cells and other connective tissue elements. Vessels within the sclerotic centre of a tumour are not included. The Chalkley eyepiece graticule increases objectivity of counting (Fox *et al*, 1995a). Computerised image analysis, which allows evaluation of several parameters in addition to microvessel counting, such as percentage area stained per field, area of vessel lumina (Fox *et al*, 1995a), and intratumoral microvessel density grading are also used (Vermeulen *et al*, 1996). Biochemical assessment of growth factors in the serum and urine of cancer patients is potentially of use in screening and provides a non-invasive method for monitoring response to treatment and identifying recurrence. Elevated levels of bFGF and VEGF have been found in the sera of patients with metastatic colorectal, breast, ovarian and renal carcinomas (Dirix *et al*, 1997). bFGF has also been found to be elevated in the urine of patients with a wide range of malignancies (Nguyen *et al*, 1994).

Colour Doppler ultrasound may have a role in the differential diagnosis of breast lesions (Cosgrove *et al*, 1990). This allows the non-invasive assessment of breast lesions: malignant lesions are more likely to be Colour Doppler positive, benign lesions negative. The potential for colour Doppler ultrasound in monitoring response of tumours to systemic therapy has yet to be ascertained and will be addressed in a small study in this thesis.

The role of these markers of tumour vascularity in monitoring patients receiving neoadjuvant systemic treatment has not been elucidated but early markers of response would be of great value. Assessment of microvessel density on sequential biopsies taken during a course of primary systemic therapy may provide such a marker.

Therapeutic applications.

An understanding of the crucial role of angiogenesis in tumour development has led the impetus to use inhibitors of aniogenesis therapeutically. Naturally occurring inhibitors include (i) protease inhibitors, the most studied group of which are the metalloproteinase inhibitors, (ii) cytokine modulators such as interleukin-1 and thalidomide, heparin-like molecules which cause inhibition of heparin bound growth factors and (iii) vascular growth factor inhibitors, such as antisense FGF (Bicknell & Harris, 1996). Antiangiogenic therapy has low toxicity as it acts specifically to inhibit proliferation of endothelial cells, demonstrated by the action of platelet factor 4 and the fumagillin derivative, AGM-1470 (TNP-470). Extensive pre-clinical studies testing these angiogenic inhibitors have led to the emergence of general principles guiding their use (Folkman, 1995).

Antiangiogenic therapy appears to have its greatest efficacy if given over a prolonged period of time. It may play an important role following primary treatment of tumours by conventional treatment modalities, when it can be given over a long period to prolong dormancy of microscopic disease. Interferon-α-2a has been used for up to one year to treat haemangiomas of infancy (Ezekowitz *et al*, 1992). Angiogenic inhibitors may be particularly useful in combination with conventional cytotoxic chemotherapy and some evidence suggests that this combined approach may be curative whilst individual agents alone are not (Teicher *et al*, 1994).

In this section the process of angiogenesis was outlined with particular attention to the relationship between oestrogen, tamoxifen and angiogenesis. Clinical applications that have developed as a result of improved understanding of the processes of angiogenesis have been discussed. It is within this background of understanding of the key role of angiogenesis in tumour growth and the potential direct effect of tamoxifen on tumour vascularity that the aims of this thesis were undertaken.

Aims of the thesis

To determine changes in breast tumour vascularity which occur during primary endocrine treatment and to assess the role of these changes as an early predictor of response to therapy.

These aims are addressed sequentially:

Firstly, reproducibility of the methodology to assess vascularity will be investigated in order to determine which methodology should be adopted in the main study.

Secondly, changes in tumour vascularity during primary endocrine therapy will be determined by assessment of pre- and post-treatment tumour specimens.

Thirdly, timing of the changes in tumour vascularity relative to changes in tumour volume will be investigated in a study of breast tumour xenografts.

Fourthly, a prospective study will be described aimed at determining whether early changes in tumour vascularity occurring within 10-14 days of starting treatment with primary tamoxifen can predict response to therapy.

In addition, the study aims to compare effects of tamoxifen on tumour vascularity with its effects on two other aspects of tumour biology: expression of vascular endothelial growth factor and the percentage of proliferating cells.

Materials and Methods

In order to achieve the aims outlined on the previous page, four main studies have been performed. Methods involved in the studies will be described in this chapter.

(i) Validation of methodology in terms of observer variation, variation due to the use of two antibodies and reproducibility of microvessel counts in different breast cancer specimens.

(ii) Changes in breast tumour vascularity following a period of neoadjuvant tamoxifen in relation to clinical response.

(iii) Sequential changes in tumour vascularity in xenografted breast cancers during tamoxifen treatment and correlation with changes in tumour volume.

(iv) Sequential assessments of tumour vascularity in patients with primary breast cancers during treatment with tamoxifen and correlation with response.

Two control experiments were also performed to compare effects of tamoxifen on other aspects of tumour biology: vascular endothelial growth factor (VEGF) expression, which is related to angiogenesis, and changes in proliferation, which are independent of it.

(v) Changes in VEGF expression following a period of neoadjuvant tamoxifen in relation to response.

(vi) Sequential assessment of proliferation in association with response to tamoxifen as a control study.

Similar methodologies have been adopted throughout the thesis, based on obtaining specimens of tumour in the form of biopsies or excision specimens. Tumour sections were immunohistochemically stained with antibodies to elements of the endothelial cell, to VEGF and to proliferating cells (MIB-1 antibody to Ki-67). Assessments were then made by microvessel counting or by the estimation of percentage tumour cells staining. Information regarding tumour response to tamoxifen was collected by clinical, ultrasonographic or mammographic assessments of changes in tumour volume. Response data were correlated with changes in vascularity, VEGF and MIB-1 expression.

Methodological details regarding tumour retrieval and assessments of response will initially be considered in four sections, corresponding to each study performed. This will be followed by a section describing aspects of the method common to all studies, including histology and immunohistochemistry. Full details of materials used are described in the Appendix to this chapter.

Validation of methodology.

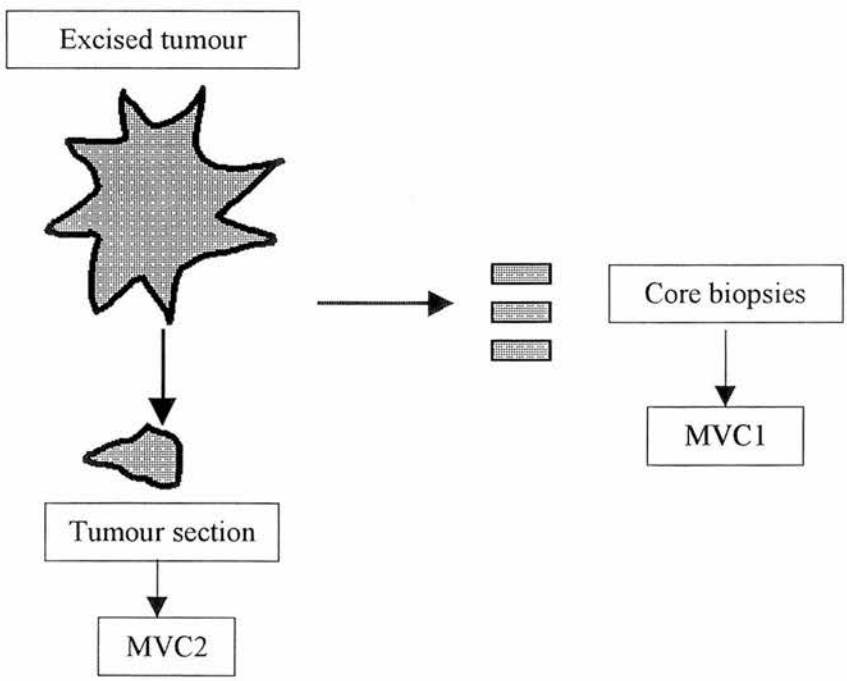
Study design:

Intra- and interobserver variation: To compare microvessel counts performed by the same observers at different times and by two different observers, microvessel counts were obtained following staining of tumour sections with an endothelial cell marker. In thirty-one tumours, counts performed by the same observers at least a fortnight apart were compared. Sixteen of these were core biopsies. Counts performed by two observers separately in sixteen tumour sections were compared.

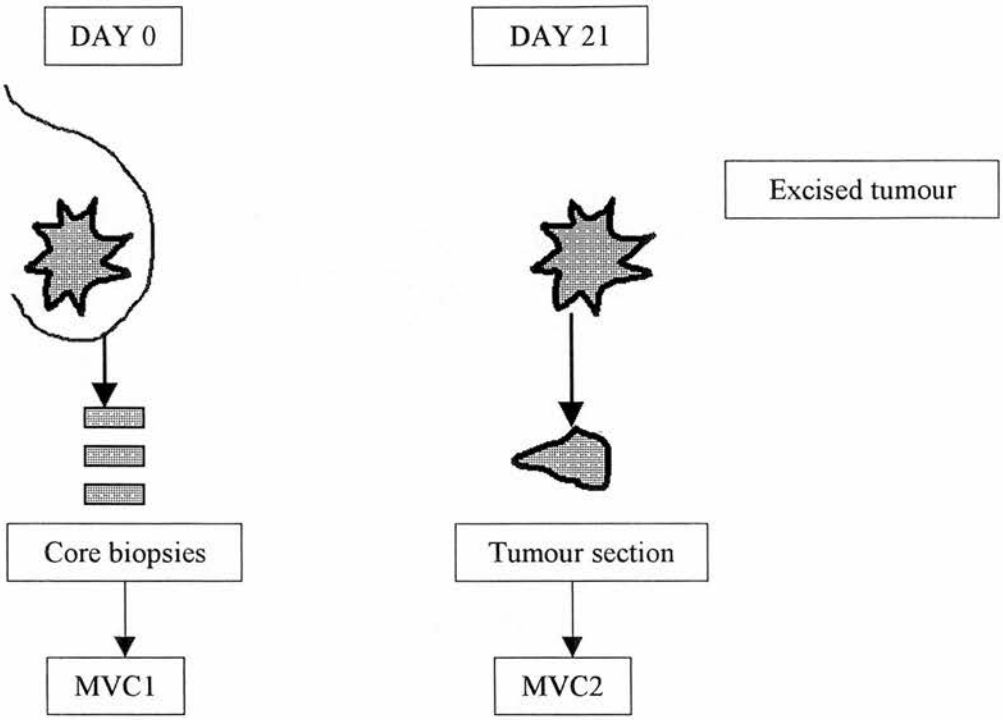
Comparison of counts obtained following staining with antibody to Factor VIII and CD31: To compare results obtained from the same tumours following staining with two different antibodies to endothelial cells, immunohistochemical staining was performed on sequential sections with antibody to Factor VIII and to CD31 (PECAM). Counts were compared in core biopsies (n=110) and tumour sections (n=164).

Reproducibility of method in different breast cancer specimens: To determine the relationship between counts obtained from different specimens of the same tumour with no intervening treatment, three studies were undertaken:

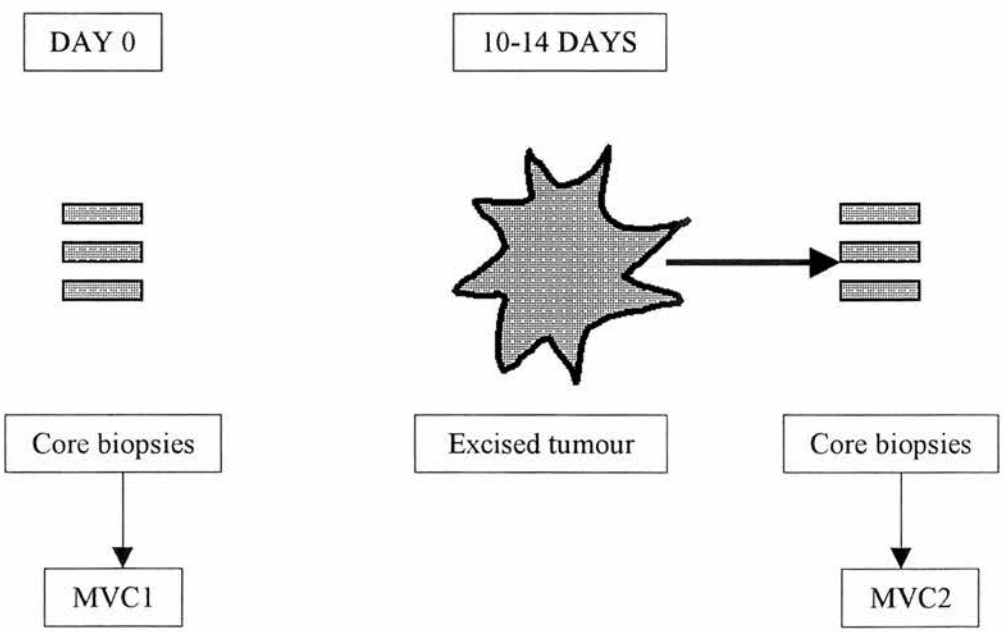
(i) Comparison of counts obtained from core biopsies (MVC1) and tumour sections (MVC2) taken immediately following tumour excision:



(ii) Comparison of counts performed on core biopsies (MVC1) and tumour sections (MVC2) taken approximately three weeks apart with no intervening treatment:



(iii) Comparison of counts performed on core biopsies obtained at two time points at least ten days apart, with no intervening treatment:



Methods:

For determination of intra-observer variation (n=31) and interobserver variation (n=16) and for comparison of counts performed following staining with two endothelial cell antibodies in core biopsies (n=110) and tumour sections (n=164), specimens collected for other aspects of the study were studied.

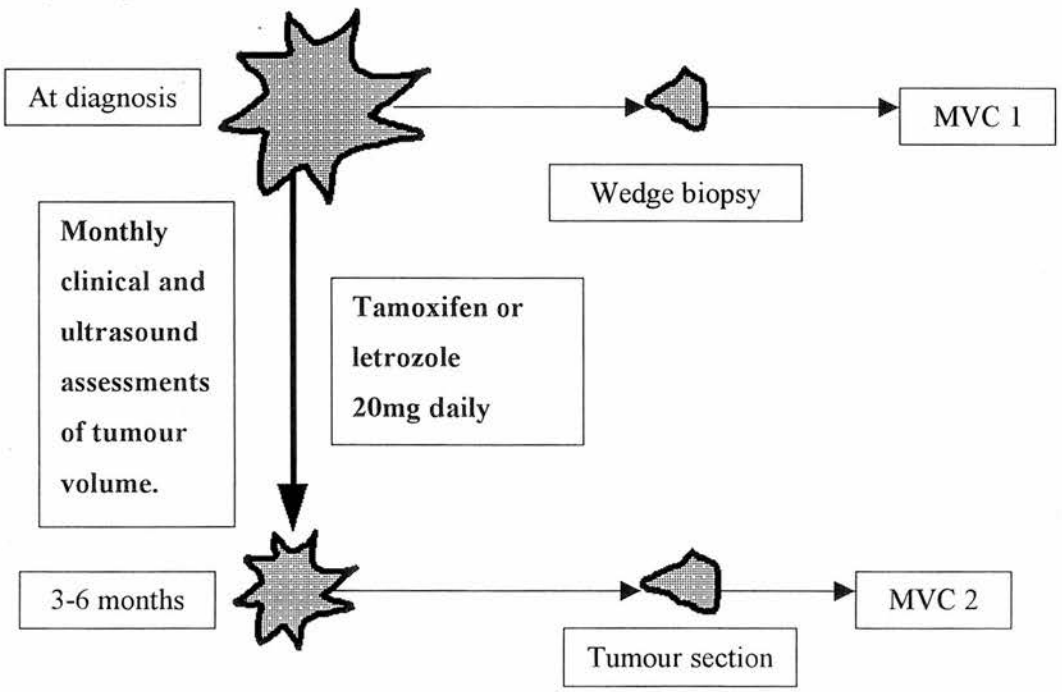
For assessment of reproducibility of methodology in different breast cancer specimens, in sixteen excised tumours, core biopsies were taken and tumour sections obtained at the same time. Twenty-one consecutive patients with newly diagnosed breast cancer underwent core biopsies for confirmation of diagnosis and determination of ER status. Following surgical excision of tumour between five and thirty-five days later, transverse tumour sections were collected. In ten additional cases, core biopsies were performed at diagnosis and repeated immediately following surgical excision, between five and twenty-five days later. Description of the process of taking core biopsies will be described in a later section.

Changes in tumour vascularity following neoadjuvant tamoxifen in relation to clinical response.

To determine whether an association existed between changes in tumour vascularity and response to primary endocrine therapy, seventy-two patients were recruited into this retrospective study. Fifty-seven of these postmenopausal women with large, operable, ER-positive disease were treated with tamoxifen for a period of 3-6 months. Tumour sections before and after treatment were stained with the endothelial cell markers, antibody to Factor VIII and CD31, and microvessel counts performed (mvc1 and mvc2). Tumours were stained with antibody to vascular endothelial growth factor (VEGF). Clinical response data were available and allowed comparison of microvessel counts in relation to response. A further fifteen patients had been treated with neoadjuvant tamoxifen for longer than six months and were analysed as a separate group.

In addition, twenty-two patients treated with neoadjuvant letrozole, an aromatase inhibitor, took part in the study as a separate group. Microvessel counts were compared before and after treatment and correlated with response as described above. This allowed comparison of effects of the two drugs on tumour vascularity in relation to response.

Study design:



Methods:

Patient recruitment: Since 1991, it has been policy at the Edinburgh Breast Unit to treat postmenopausal women with large (>3cm diameter), operable breast tumours or locally advanced disease (fixed ipsilateral axillary lymph nodes or involvement of skin or muscle) with primary systemic therapy. Those patients with oestrogen receptor (ER)-rich disease (defined as greater than 20fmol/mg cytosolic protein) were treated with tamoxifen (20mg daily) following a wedge biopsy, performed under local anaesthetic, for a minimum period of three months. Tumour response in these patients was monitored by monthly clinical and ultrasound measurements of tumour volume. On completion of tamoxifen treatment patients underwent wide local excision or mastectomy. Seventy-two patients identified by casenote review have been included in the study. Patients were excluded if there was insufficient information regarding clinical response and if pre- and post- treatment tumour tissue was not available from the archives of the Pathology department.

Tumour retrieval: All patients had undergone wedge biopsy following diagnosis of breast cancer during the period of September 1991 and July 1996. Wedge biopsies had been performed under local anaesthetic as day case surgery. On completion of a period of neoadjuvant tamoixfen, patients underwent definitive surgery in the form of wide local excision or mastectomy with axillary surgery. Tumour sections had been stored in neutral-buffered formalin or methacarn for 24 hours and were then paraffin-embedded. Patients eligible for participation in the study were identified by casenote review. Sections stained with haematoxylin and eosin were retrieved from the Pathology archives and reviewed by a single pathologist for identification of the block containing the most representative tumour area. Between fifteen and thirty consecutive 4μm sections were cut from that block and mounted on Superfrost microscope slides.

Monitoring of clinical response to tamoxifen: Response data were obtained from the casenotes. Patients were monitored by monthly clinical and ultrasound assessments of tumour volume. In addition, they underwent mammography before and after treatment. A responding tumour was defined as one in which there was a greater than 25% reduction in ultrasound volume over the 3 month period. If information on ultrasound assessment was incomplete, but there was clear documentation of response in the notes, the patient was included. Patients in whom such information was insufficient to allow clear definition of

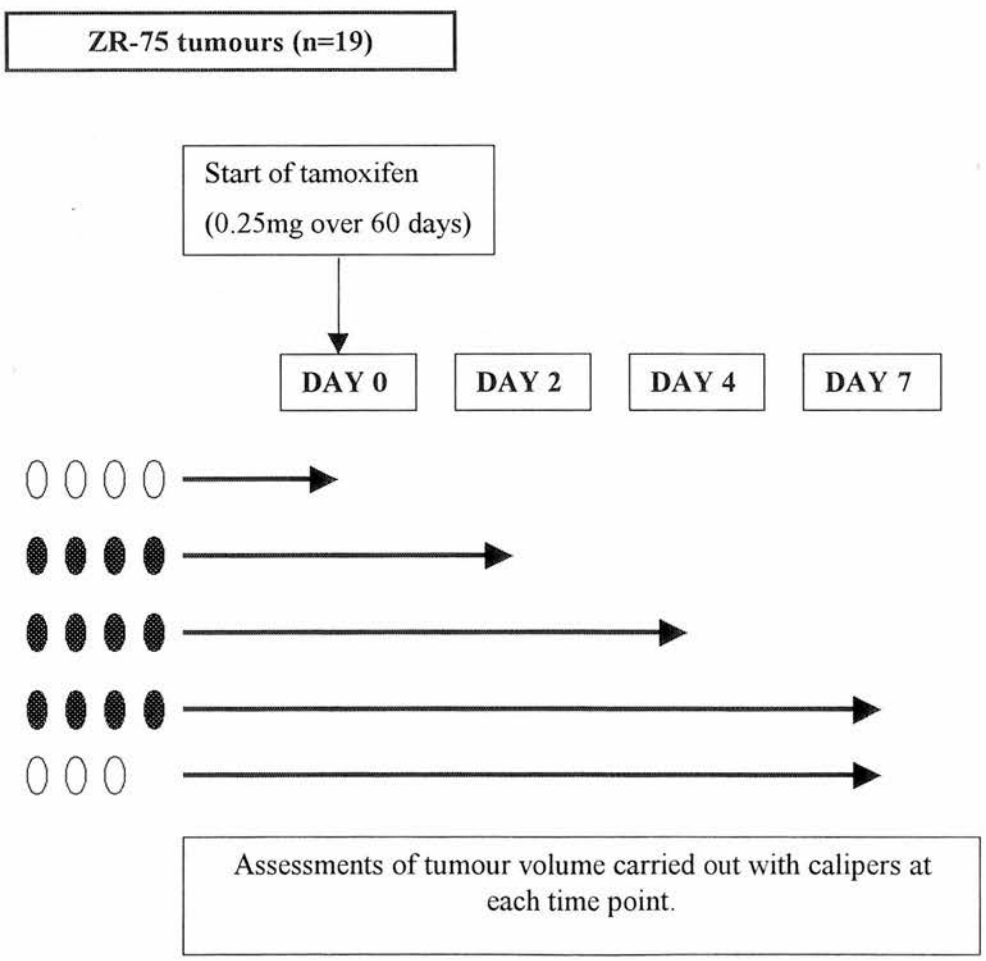
response were excluded. The methods of clinical and ultrasound assessment will be outlined in the description of the prospective study.

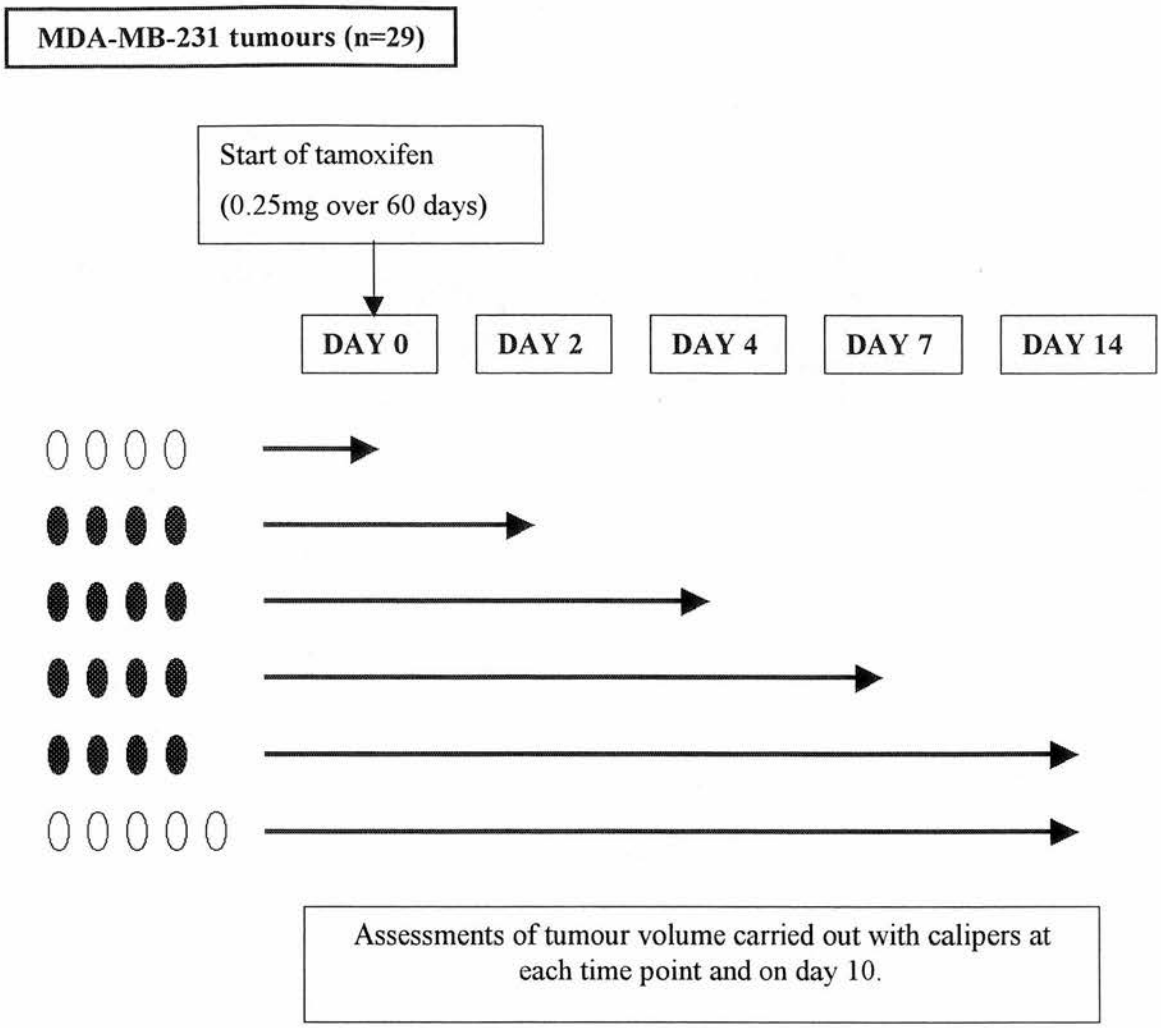
Sequential changes in tumour vascularity in xenografted breast cancers during tamoxifen treatment and correlation with changes in tumour volume.

To determine chronology of changes in tumour vascularity during primary tamoxifen treatment relative to tumour response, ER-positive (ZR-75) and ER-negative (MDA-MB-231) tumours were xenografted in nude mice and animals treated with tamoxifen. Tumours were excised at several time points during treatment and sections stained with antibody to endothelial cells (MEC13.3). Microvessel counts were then performed on stained sections and correlated with changes in tumour volume with treatment.

Study design:

Where ○ represents a control tumour and ● represents a treated tumour.




MDA-MB-231 tumours (n=29)
Start of tamoxifen
(0.25mg over 60 days)
DAY 0
DAY 2
DAY 4
DAY 7
DAY 14
Assessments of tumour volume carried out with calipers at each time point and on day 10.

Methods:

Tumour growth: The oestrogen-sensitive ZR-75 (Engel *et al.*, 1978) and oestrogen-insensitive MDA-MB-231 (Cailleau *et al.*, 1974) breast cancer cell lines were maintained by sequential passage in nude athymic mice (HsdOla: ICRF-nu), purchased from Harlan (UK), Bicester, Oxon. At each passage a 1mm fragment of tumour was inserted subcutaneously in flanks of animals when the mice were 8-12 weeks old.

On the day of implantation, a slow release oestrogen pellet (0.72mg, released over 60 days, supplied by Innovative Research of America, Ohio, USA) was inserted subcutaneously in the midline. All mice were maintained on a standard diet (Harlan, UK) and water ad libitum. Bidimensional tumour dimensions were recorded weekly, and tumour volumes calculated according to the equation:

$$\text{VOLUME} = D \times d^2 \times \pi/6$$

Where D was the larger of the two diameters.

When tumour volumes reached an average of $0.25cm^3$, animals were randomly allocated to treatment or control groups (Day 0). Animals in treatment groups were treated with a slow release tamoxifen pellet (0.25mg over 60 days, Innovative Research of America), inserted subcutaneously in the midline, adjacent to the oestrogen pellet. Animals in control groups had no further pellet inserted.

Tumour retrieval: Animals were killed by cervical dislocation and tumours excised on days 0, 2, 4 and 7 in the ZR-75 group and days 0, 2, 4, 7 and 14 in the MDA-MB-231 group. Numbers of tumours excised at each time point were illustrated in the study design. Tumours were divided in half immediately following excision and were stored in either liquid nitrogen or in neutral-buffered formalin. Tumours in neutral-buffered formalin were processed in the routine pathology laboratory into paraffin-embedded blocks. 4μm sections were cut from these blocks and mounted on Superfrost glass slides.

Response to tamoxifen: Following commencement of the experiment on Day 0, caliper assessments of transverse and anteroposterior tumour dimensions were measured on days 0, 2, 4 and 7 in the ZR-75 group and days 0, 2, 4, 7, 10 and 14 in the MDA-MB-231 group. Tumour volumes were calculated from the dimensions as described above.

Percentage response of tumours to tamoxifen was calculated at each time point, and was defined as % reduction in volume relative to the initial volume:

$$\% \text{ RESPONSE} = V_1 - V_0 / V_0 \times 100$$

Where V_0= initial tumour volume on Day 0

V_1= volume at later time point.

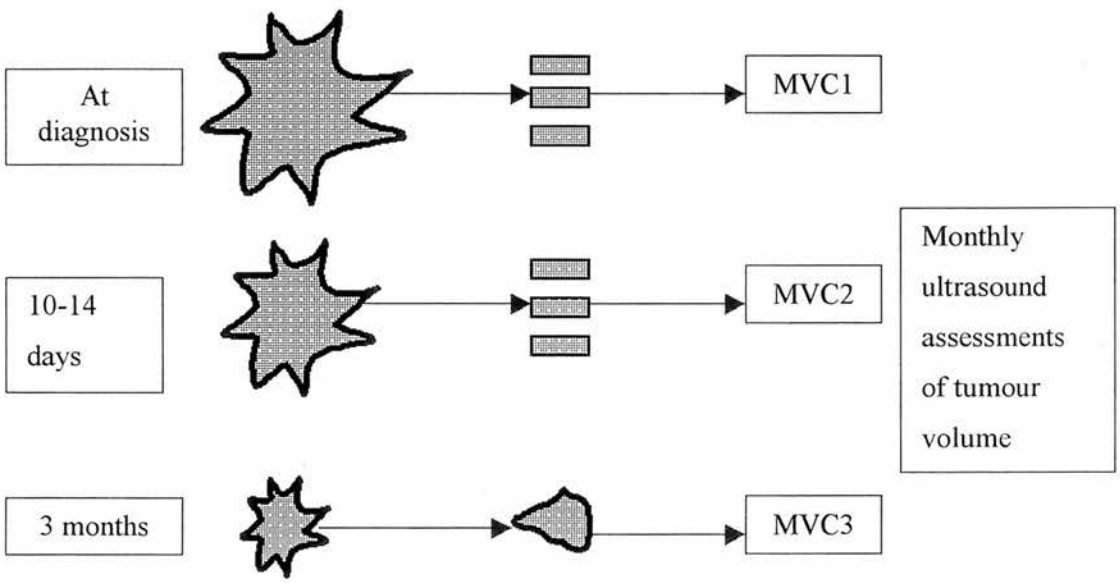
This allowed comparison of mvc in treated and control tumours. In particular, relative timings of effects of tamoxifen on mvc and tumour volume were compared.

Sequential assessments of tumour vascularity during treatment with tamoxifen and correlation with response.

To determine whether early changes in tumour vascularity predict response to primary tamoxifen treatment, forty-two patients were recruited into this prospective study. Postmenopausal women with large, operable or locally advanced, ER-positive breast cancer were treated with three months of tamoxifen prior to surgery. Patients underwent core biopsies at diagnosis and following two weeks of treatment. At the end of three months, patients underwent definitive surgery or repeat core biopsies. Assessments of tumour vascularity were thus obtained before (MVC1), during (MVC2) and after (MVC3) treatment. Response to tamoxifen was assessed by serial ultrasound measurements of tumour volume. This prospective study design allowed comparison of early changes in tumour vascularity in responding and non-responding tumours.

To compare the effects of tamoxifen on another aspect of tumour biology, sections from the same tumours were stained with an antibody to proliferating cells (MIB-1 antibody) changes in proliferation were correlated with response.

Study design:



Methods:

Patient recruitment: Having obtained written or verbal consent, fifty-two postmenopausal women with large, operable or locally advanced ER-positive breast carcinoma were recruited into a study of changes in tumour vascularity during primary tamoxifen treatment. The decision to include an individual in the study was made at the weekly multidisciplinary Combined Breast Clinic. In addition to the criteria described, tumour dimensions had to be assessable on ultrasound scan. Presence of regional or distant metastases did not exclude patients from the study. Patients underwent serial core biopsies before, during and after a period of three months' tamoxifen treatment and serial assessments of change in tumour volume, allowing correlation of changes in vascularity with response.

Tumour retrieval:

Core biopsies: These were performed under local anaesthetic in the clinic at the time of diagnosis and repeated following two weeks of treatment with tamoxifen. In those patients who continued with tamoxifen beyond three months, core biopsies were repeated at that time. Having infiltrated the area within and around the tumour with 20mls. 1% lignocaine and 1:200 000 adrenaline, a single stab incision was made at a point lateral to the tumour with a Size 15 scalpel blade. Biopsies were carried out using a Pro-Mag gun and a 14 gauge needle, illustrated on Figure 1a, which was inserted at the point of incision and biopsies taken by placing the tip of the needle at the tumour periphery and firing the gun into the tumour (Figure 1b). Between one and ten core biopsies were collected from each tumour. A single core biopsy is illustrated in Figure 1c. Biopsy specimens were placed immediately into neutral-buffered formalin and processed into paraffin-embedded blocks within 24 hours.

Tumour sections: Immediately following surgical excision, fresh tumour was collected and, in conjunction with the pathologist, a transverse tumour section was obtained for the purposes of this study. Specimens contained both central and peripheral tumour areas and were stored immediately in neutral-buffered formalin. Specimens were processed in the routine pathology laboratory and were paraffin-embedded within twenty-four hours of collection. Blocks were assigned a numerical code to allow blind assessment.

Fig 1

a) Pro Mag gun with 14 gauge needle

b) Core biopsy of a breast tumour performed under local anaesthetic.

c) An example of a core biopsy.

Monitoring of tumour response to tamoxifen:

Serial assessments of tumour volume were made by clinical and ultrasound measurements. In addition, patients underwent mammography before and after treatment. A subgroup of nine patients also underwent serial colour Doppler ultrasound scans.

Clinical measurements: These were made using calipers to measure tumour dimensions. Transverse and anteroposterior measurements were obtained at diagnosis and following six and twelve weeks of treatment.

Ultrasound measurements: All ultrasound scans were performed by a single observer (L.M.) at diagnosis, and repeated following six and twelve weeks of treatment. Three tumour dimensions were assessed: the maximum transverse and anteroposterior diameters and tumour depth. Tumour volumes were calculated according to the equation:

$$\text{VOLUME} = A \times B \times C \times \pi/6$$

Where A= transverse diameter

B= anteroposterior diameter

C= tumour depth.

Percentage response of tumours to tamoxifen was calculated at each time point, and was defined as % reduction in volume relative to the initial volume:

$$\%\ \text{RESPONSE} = V_1 - V_0 / V_0 \times 100$$

Where V_0= initial tumour volume

V_1= volume at later time point.

A responding tumour was defined as one in which there was progressive reduction in tumour volume which, at the end of three months, represented a greater than 25% decrease compared with initial tumour volume.

Colour Doppler ultrasound: Scans were performed by one of two consultant radiologists in a subgroup of nine patients. Tumour dimensions were measured as described for conventional ultrasound and the largest feeding vessel was identified following scanning of the entire tumour. A colour Doppler waveform was obtained and maximum systolic velocity determined as the peak velocity of the waveform. This provided non-invasive assessments of tumour vascularity for comparison with subsequent assessments during the treatment period.

Common methodology:

Histology:

A single section of each tumour was stained with haematoxylin (Sigma) and eosin (Sigma). This was performed in the routine pathology laboratory.

Immunohistochemistry:

Endothelial cell markers:

Sections of all clinical and xenografted tumours were stained with an endothelial cell marker highlighting tumour vessels. In this section, a description of the final methods of staining will be given, followed by explanation of methods used to determine optimal staining method for each group of tumours. Specific staining protocols are provided in the Appendix.

Clinical tumour sections were stained with two monoclonal antibodies to endothelial cells: anti-Von Willebrand factor (antibody to Factor VIII) (DAKO) and mouse anti-human CD31 antibody. Similar techniques of antigen retrieval, secondary antibody and visualization were used for both antibodies. The optimum concentration of anti- Von Willebrand factor (VWF) was 1: 80, diluted in 20% fetal calf serum (FCS, Gibco) / 0.05M tris-buffered saline (TBS). TBS was made up with 60.55g Trizma base (Sigma) and 1 litre distilled water. This was optimized to pH7.6 with concentrated hydrochloric acid and then diluted to 0.05M with normal saline (100mls TBS: 900mls 0.9% sodium chloride). Optimum concentration of antibody to CD31 was 1:40, diluted as for anti-VWF.

Xenograft tumours were stained with rat anti-mouse CD31 monoclonal antibody (MEC 13.3), adopting a similar protocol as for clinical tumours. Optimum primary antibody concentration was higher than for clinical tumours: 1:10 diluted in 20% FCS/0.05M TBS.

Following dewaxing of sections through graded xylene, described in detail in the Appendix, sections were incubated in distilled water warmed to 37°C in an incubator. Antigen was retrieved using protease (Type XXIV, Sigma). 25mg protease was dissolved in 200ml warm phosphate-buffered saline (PBS, Dulbecco), and slides were incubated in the solution for 20 minutes.

Following washing of slides in 20% fetal calf serum in 0.05M TBS, primary antibody was applied at the optimum concentration. Tumour sections were incubated for 1 hour at room temperature or overnight at 4°C and were then washed in 0.05M TBS.

The secondary antibody used for clinical tumours was rabbit-anti-mouse immunoglobulin (DAKO). This was diluted 1:50 in 0.05M TBS and sections incubated for between 30 minutes and one hour. The secondary antibody used for xenograft tumours was rabbit-anti-rat immunoglobulin (DAKO) at 1:10 in 0.05M TBS. After incubation of slides with secondary antibody, they were washed in TBS and mouse alkaline phosphatase/anti-alkaline phosphatase (APAAP, DAKO) was applied for 30 minutes to clinical tumours (concentration: 1:100 in 0.05M TBS). Rat APAAP was used as a tertiary antibody in xenograft experiments (concentration: 1:50 in 0.05M TBS).

Application of the secondary and tertiary antibodies was repeated to enhance staining for 10 minutes each. Following washing in TBS for 5 minutes, new fuchsin substrate (DAKO) was applied to sections for 20 minutes. Sections were then washed in distilled water and counterstained in haematoxylin (Sigma) for 5 minutes with differentiation through acid-alcohol (0.1% hydrochloric acid in 400mls 95% alcohol) and lithium carbonate (Sigma). Sections were rinsed in running tap water and mounted immediately in aqueous mounting medium (BDH) using glass coverslips (Mackay and Linn). Stained sections were stored at 4°C.

Summary of methods used:

Antibody	Antigen retrieval	Dilution/ Diluent	Incubation	Secondary antibody	Tertiary reagent
CD31	Protease	**1:40** 20%FCS in TBS	Room temperature, 1 hour	Rat anti-mouse	Mouse APAAP
FACTOR VIII	Protease	**1:80** 20%FCS in TBS	Room temperature, 1 hour	Rat anti-mouse	Mouse APAAP
MEC 13.3	Protease	**1:10** 20%FCS in TBS	Room temperature, 1 hour	Rabbit anti-rat	Rat APAAP

Optimization of immunohistochemistry:

Several attempts were made to optimize endothelial cell staining of both clinical and xenograft tumours. Particular problems were faced with xenograft tumours, in which initial attempts at staining failed, yielding either negative results or non-specific staining. Thus several methods other than those described in the previous section were tried for each stage of the staining process and these will be described. The optimized staining technique was the one in which clear red staining of vessels was achieved with minimal background staining.

Antigen retrieval: Methods other than retrieval with protease were adopted, involving microwave in citric acid and various methods of trypsin antigen retrieval.

Microwaving in citric acid: sections were placed in a solution of 1.07g citric acid in 500ml distilled water. Concentrated hydrochloric acid was added to the solution to reach pH6.0. Sections were microwaved for 3x5 minutes at full power and allowed to stand in citrate for 20 minutes.

Trypsin: several methods were attempted for the xenograft tumour slides:

Sections were placed in a solution of 0.1% trypsin in 0.1% calcium chloride at pH7.4 for 30 minutes at 37°C and washed in water.

Sections were placed in tissue culture trypsin at either room temperature or at 37°C for varying lengths of time for optimization (20-50 minutes).

Primary antibody concentrations: These were varied for each antibody to determine optimal concentrations, in which clear red staining of vessels was obtained with minimal background staining. Range of concentrations used was 1:10-1:200. Optimal concentrations have been described.

Secondary antibodies: for xenograft tumours, use of two secondary antibodies was attempted; both were rabbit-anti-rat immunoglobulins, one linked into the Avidin-Biotin complex (ABC) and the other into the APAAP system. Concentrations were varied and the antibody linking into the APAAP system found to provide better staining than that linking into the ABC system.

Tertiary systems: As has been described, the ABC complex was used in an attempt to improve staining of xenograft tumours. Following application of the secondary antibody the APAAP step was replaced by application of the ABC complex. Visualization following use of the ABC complex was achieved with diaminobenzadine (DAB). Using this technique, sections were then dehydrated through graded alcohols in the reverse manner to that described for hydration and mounted using DPX mounting medium.

Antibody to vascular endothelial growth factor (VEGF):

Tumour sections of fifty-seven patients before and after treatment with tamoxifen were stained with antibody to VEGF. This work was carried out in collaboration with Dr. J. Harmey at the Royal College of Surgeons of Ireland, Dublin. Tumour sections were obtained by the investigator and all staining performed by Miss. D. Foley in Dublin. Assessment of staining was performed jointly by Dr. J. Harmey and the investigator.

4μm sections mounted on Superfrost slides were dewaxed as for the previous experiment. Sections were rinsed in running water and then in 0.05M TBS. Sections were incubated in 3% hydrogen peroxide for 5 minutes (Sigma) to quench endogenous peroxidase activity (3mls Hydrogen peroxide in 100mls distilled water). This step was repeated, before rinsing slides in running water, then placing in 0.05M TBS.

Slides were incubated in normal goat serum (DAKO) diluted 1:10 in TBS for 30 minutes to block non-specific binding. Excess serum was wiped off before incubation of sections in primary antibody. Primary antibody was polyclonal rabbit anti-VEGF (Santa Cruz Biotechnology) diluted 1:100 in 0.05M TBS. Sections were incubated in primary antibody overnight at room temperature. Sections were rinsed in 0.05M for 5 minutes.

Secondary antibody, biotinylated goat anti-rabbit (StrepABComplex/HRP Duet mouse/rabbit kit) (DAKO) was prepared according to the kit instructions and sections were incubated for 30 minutes at room temperature. Sections were rinsed in 0.05M TBS. Sections were then incubated in StrepABComplex/ horseradish peroxidase as instructed in Duet kit for 30 minutes and rinsed in TBS.

Visualization was achieved with the chromogenic substrate diaminobenzadine (DAB). The solution was made up from two DAB tablets dissolved in 20mls. Distilled water and then aliquoted into 2ml. lots and stored frozen at -20°C. When required, a 2ml aliqout was defrosted and 2μl hydrogen peroxide added. The resulting solution was applied to sections for 5 minutes, followed by rinsing in running water. Sections were dehydrated through graded alcohols and xylene, as detailed in the Appendix. Sections were then mounted in DPX (Fisher) and dried before visualizing under light microscopy. Cells staining for VEGF appeared brown.

MIB-1 antibody to proliferating cells:

A similar method was used for staining with MIB-1 antibody, directed against proliferating cells. Following blocking of endogenous peroxidase with hydrogen peroxide solution as described previously, antigen retrieval was performed by microwaving (2 x 5 minutes at full power) in citric acid (Sigma) for 10 minutes (1.07g in 500ml distilled water). For this method a routine Sequenza system was used. The primary antibody was MIB-1 (Biogenex) diluted to 1:20 with 0.05M TBS. Slides were incubated in primary antibody for 30 minutes, then rinsed in PBS for 5 minutes. Slides were incubated in Vector anti-mouse secondary antibody at a concentration of 1:200 in 0.05M TBS for 30 minutes and rinsed in PBS. Tertiary antibody was applied for 30 minutes followed by visualization with DAB as previously described. Sections were counterstained with haematoxylin for 5 minutes and then dehydrated through alcohols and xylene. Sections were mounted in DPX.

Microvessel counts:

Following immunohistochemical staining of endothelial cells as described, microvessel counts were performed by two observers, a trainee pathologist and the investigator, who underwent a period of training with an expert in the field. Counts were performed using a conference microscope.

An H+E section of the block was initially scanned and the tumour identified. Having scanned the tumour at low power (x40 and x100) the area of highest vascularity was identified, known as a vascular 'hot spot'. A count was then performed in this area under high power (x250), using a Chalkley 25-point eyepiece graticule (Fox *et al*, 1995a). This graticule with 25 randomly allocated dots was placed in the microscope eyepiece. Vessels were counted only if they lay under the dots. In order to account for volume occupied by vessels, those with more than one dot overlaying them were counted more than once. Counts were performed in three vascular hot spots. The highest, mean and total of three counts were documented. All sections were labelled with a number and those performing the counts had no knowledge of the patient's response or whether the section was taken prior to or after treatment.

VEGF assessment:

Following staining with antibody to VEGF sections were assessed by three observers using a conference microscope: by Miss. D. Foley, Dr. J. Harmey and by the investigator. Assessments were made on intensity of staining and on percentage of tumour cells staining positive for VEGF and scored as described on Table 2.1. Assessments were made in three random high power (x400) fields and the total of three scores were documented. Intensity of staining was compared to the positive control for each staining run.

Table 2.1a: Scoring categories for antibody to VEGF-intensity:

SCORE	INTENSITY
0	No cells
1	Weak
2	Moderate
3	Strong

Table 2.1b: Scoring categories for antibody to VEGF-% cells staining:

SCORE	% CELLS STAINING
0	<1
1	1-25
2	26-50
3	51-75
4	76-100

MIB-1 assessments.

Assessments were performed by a single observer, Dr. S. Iqbal; in one in four sections, scores were repeated by Dr. T. Anderson and any discrepancies in the scores between the two observers were discussed. Scores were based on percentage of tumour cells stained for MIB-1 as described in Table 2.2. Scores were obtained by scanning the entire tumour section.

Table 2.2: Scoring categories for MIB-1 assessments:

SCORE	% CELLS STAINING
0	0
1	1
2	1-10
3	11-33
4	34-66
5	>67

Data and statistical analysis:

All data were stored on an Excel for Windows version 5.0 database:
(Microsoft Corporation, Redmond, WA, USA).

Statistics were performed using InStat program for MacIntosh computers. Comparison of microvessel counts between non-responding and responding tumours were assessed using the non-parametric Mann-Whitney test. The paired Wilcoxon signed rank test was applied for comparison of counts within the same group of tumours at different times. When determining trends of change in microvessel count with treatment, Chi-squared test for trend was used. Finally, the Spearman rank correlation test was applied for determination of correlation between counts and/or other factor.

Results

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1.2 Comparison of antibodies to Factor VIII and CD31.

1.3 Tumour heterogeneity.

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2.3 Microvessel counts: antibody to CD31.

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4.2 Microvessel counts following staining with antibody to Factor VIII.

4.3 Microvessel counts following staining with antibody to CD31.

4.4 Comparison of microvessel counts performed following staining with antibodies to Factor VIII and CD31.

4.5 Microvessel counts and other tumour parameters.

4.6 Colour Doppler ultrasound and assessment of tumour vascularity.

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5. Changes in %tumour cells expressing vascular endothelial growth factor (VEGF) and tamoxifen treatment.

5.1 % VEGF-positive tumour cells and response.

5.2 % VEGF-positive tumour cells and microvessel counts.

5.3 Discussion.

6. Changes in tumour cell proliferation during tamoxifen treatment.

6.1 Proliferation scores and response.

6.2 Discussion.

1. Reproducibility of microvessel counts in different breast cancer specimens

Certain methodological issues regarding tumour vascularity assessment by microvessel counting need to be addressed. Those considered in this chapter are:

Inherent variation in the method: intra-and inter-observer variation.

Variation due to selection of different antibodies to endothelial cells.

Variation due to tumour heterogeneity.

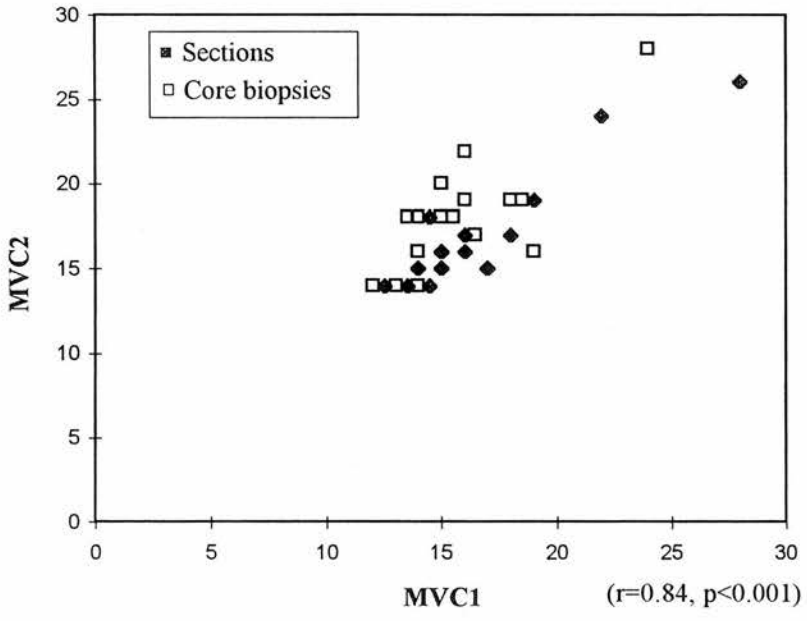
Effects of previous surgical manipulations on vascularity.

1.1. Observer variation.

Intra-observer variation.

Microvessel counts performed by the same observers on two occasions (mvc1 and mvc2) at least a fortnight apart were compared in 31 tumours, of which sixteen were core biopsies. The results are shown in Figure 1.1.1. Mvc1 ranged from 12 to 28 (median: 16, mean: 16.4) and mvc2 from 14 to 28 (median: 17, mean: 17.7). There was significant correlation between the two counts ($r=0.84$, $p<0.001$), with a tendency for the later count (mvc2) to be higher than the first count (mvc1). The standard deviation of difference between the two groups was 1.95, giving an expression of variance of 11%. Data are detailed in the appendix to this chapter.

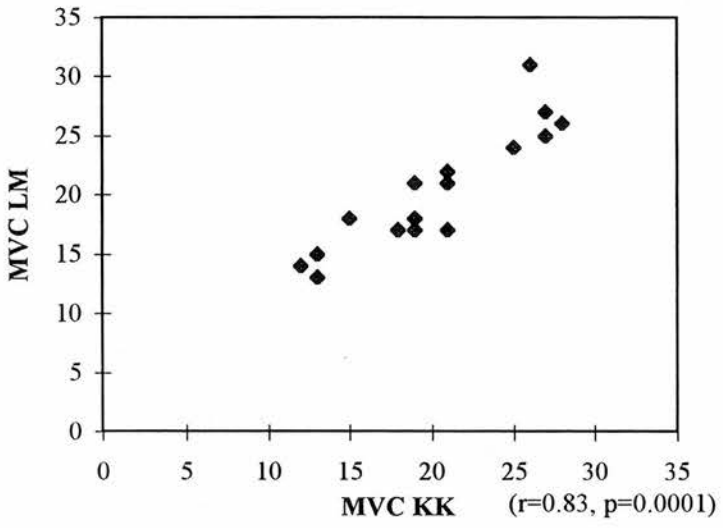
Figure 1.1.1: Correlation of counts at different time points,



Inter-observer variation.

Microvessel counts were performed by two different observers (mvcKK and mvcLM) in 16 tumours, all of which were tumour sections. MvcKK ranged from 12 to 28 (median: 20, mean: 20.3), mvcLM ranged from 13 to 31 (median: 19.5, mean: 20.5) as shown in Figure 1.1.2. A significant correlation was found between counts of the two observers ($r=0.83$, $p<0.001$). The standard deviation of differences was 3.03, giving an expression of variance of 15%.

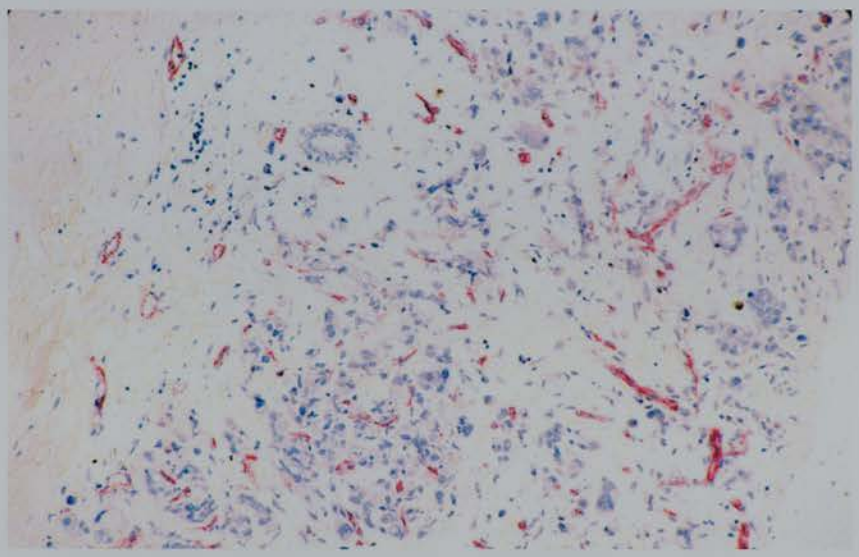
Figure 1.1.2: Correlation of counts performed by two observers.



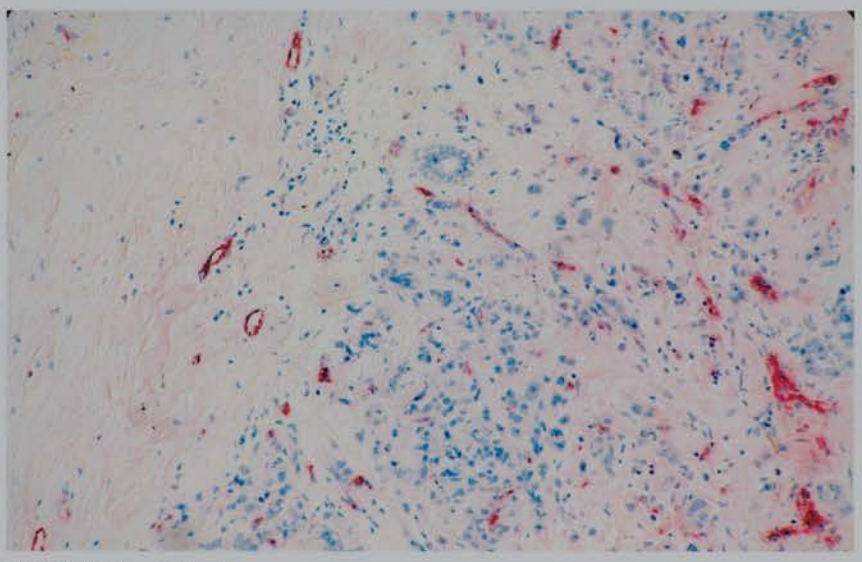
1.2. Comparison of mvc in core biopsies and tumour sections following staining with CD31 and FVIII antibodies.

Microvessel counts were performed following endothelial cell staining with two different antibodies: antibody to CD31 and to Factor VIII in both core biopsies (n=110) and tumour sections (n=164) for comparison, illustrated on Figure 1.2.1. The relationships between counts performed on serial sections are shown on Figures 1.2.2 and 3. There was a highly significant correlation in counts using different antibodies in both cores ($r=0.40$, $p<0.0001$) and tumour sections ($r=0.30$, $p=0.0001$).

Fig 1.2.1. Comparison of endothelial cell staining with antibody to factor VIII and CD31 with vessel highlighted in red.



a) Factor VIII antibody



b) CD31 antibody

Figure 1.2.2: Relationship between mvc performed on core biopsies following staining with CD31 and FVIII antibodies.

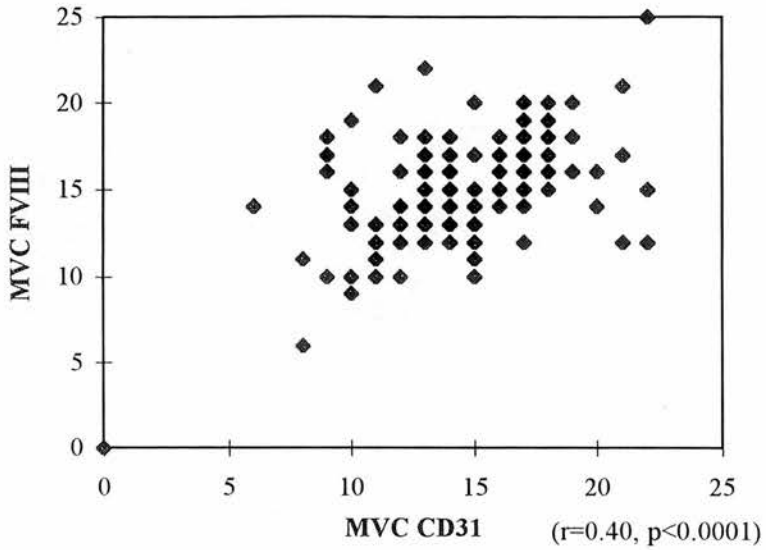
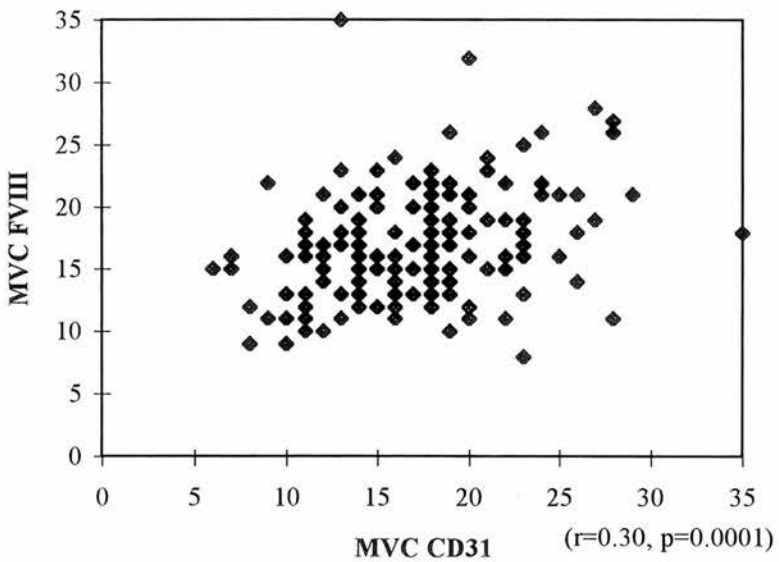


Figure 1.2.3: Relationship between mvc of tumour sections following staining with CD31 and FVIII antibodies.

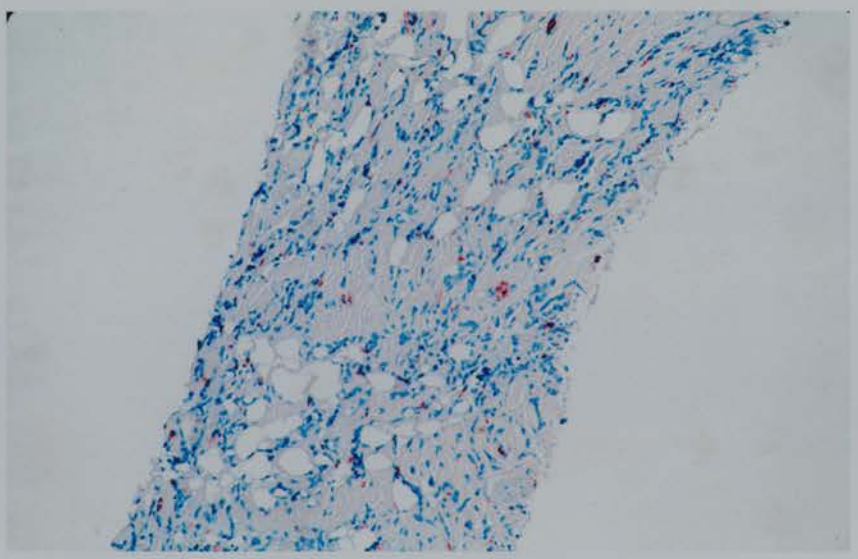


1.3. Tumour heterogeneity.

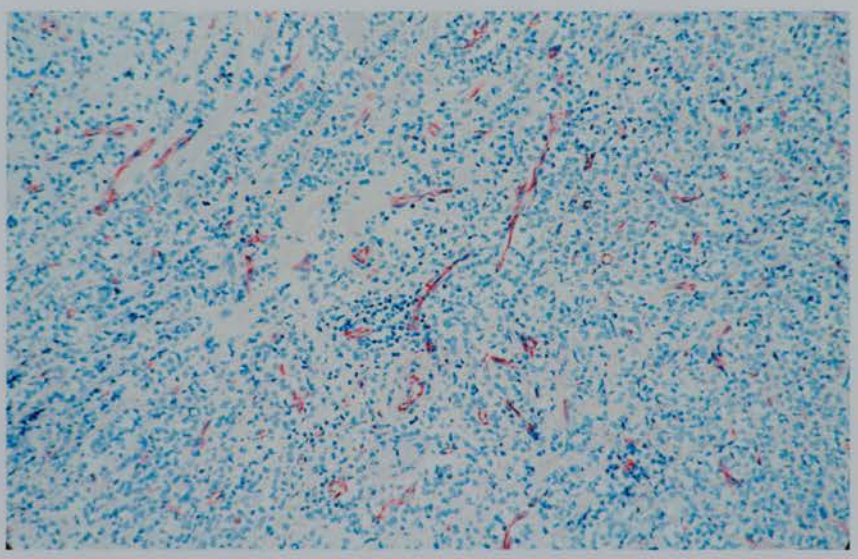
Comparison of mvc in core biopsies and tumour cross-sections.

Core biopsies were taken of 16 tumours immediately following surgical excision and counts were compared in cores and tumour sections, illustrated in Figure 1.3.1. The number of core biopsies taken from each tumour ranged from 1 to 7 (median: 3, mean: 3.33); all biopsies taken were mounted on a single slide and scanned at low power to identify vascular 'hot spots'.

Fig 1.3.1



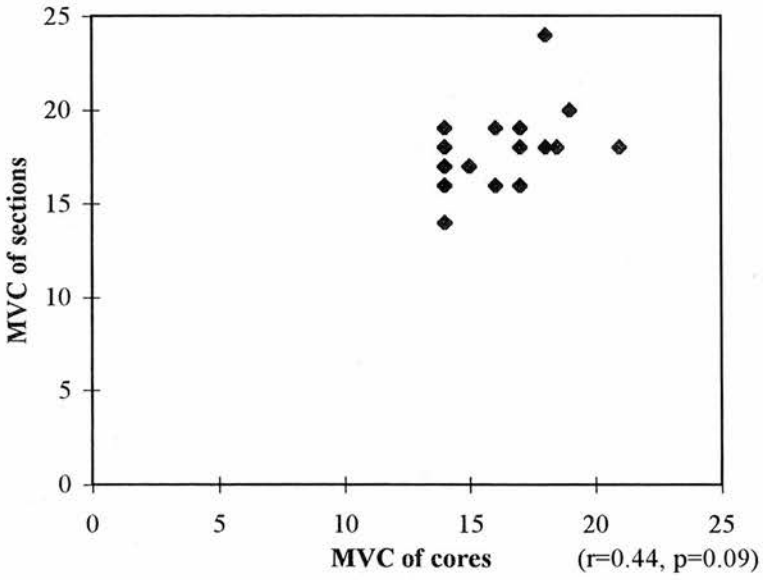
a) Endothelial cell staining of section obtained from core biopsy.



b) Endothelial cell staining of tumour cross section

Figure 1.3.2 illustrates the correlation between counts in cores and tumour sections. Mvc in core biopsies ranged from 13 to 22 (median: 17, mean: 16.53) and in tumour sections from 14 to 24 (median; 18, mean: 18). There was no significant difference in mvc between the two groups (p=0.1, paired Wilcoxon). Conversely, whilst there was a positive correlation in counts, this did not reach statistical significance (r=0.44, p=0.091). In nine of sixteen tumours (56%) mvc was higher in the tumour cross-section whereas it was higher in the core biopsies in three of sixteen (19%) with no difference in 4 (25%).

Figure 1.3.2: Relationship between mvc of cores and tumour sections.

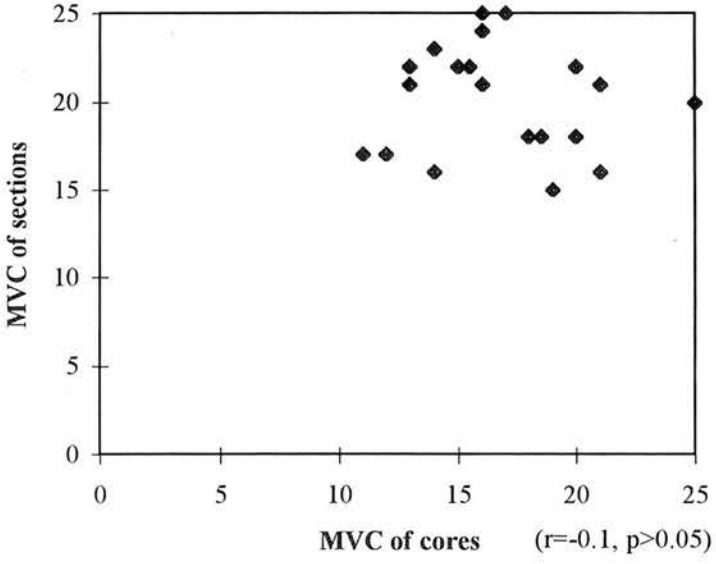


Comparison between mvc of core biopsies and subsequent tumour cross-sections.

Core biopsies were taken for diagnosis in 21 patients who underwent surgery between 5 and 34 days later (median time to surgery: 23 days) with no intervening treatment. One patient was found to have carcinoma in situ with no evidence of invasion and was excluded from the study. The number of core biopsies taken ranged from 1 to 6 (median: 3, mean: 2.95). Transverse sections of the same tumours were taken at surgery.

The relationship between counts of cores and subsequent tumour cross-sections is shown on Figure 1.3.3. Microvessel counts in core biopsies ranged from 12-25 (median:16, mean:17.1) and in the later tumour cross-sections from 15-25 (median: 21, mean: 21.1). Although there is a tight range of mvc values, no positive correlation was found (r=-0.1). There was a significant difference in the counts (p=0.0065, paired Wilcoxon). Mvc in tumour sections was higher in 13 of 20 tumours (65%), higher in core biopsies in 4 of 20 tumours (20%) and the two were the same in 3 tumours (15%).

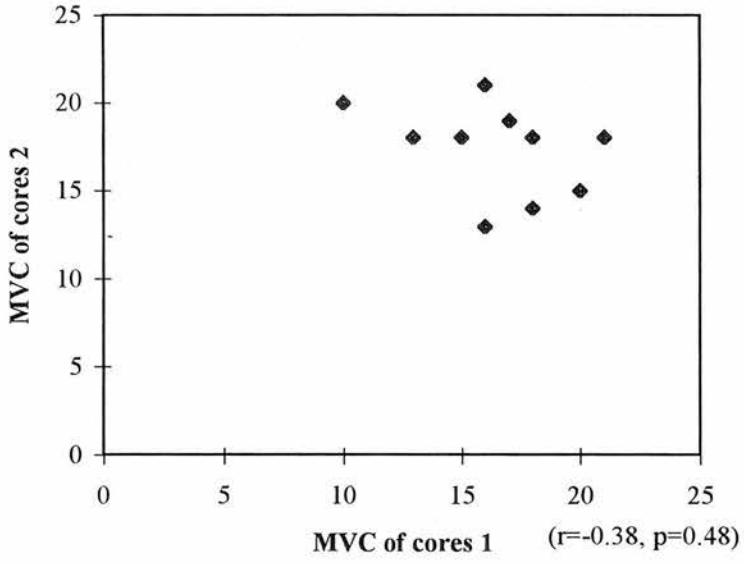
Figure 1.3.3: Relationship between mvc of cores and subsequent sections.



Comparison between mvc of core biopsies at diagnosis and following surgical excision.

Multiple core biopsies were taken of tumours in ten patients for diagnosis and were repeated immediately following surgical excision between two and three weeks later with no intervening treatment. The number of cores performed ranged from 1-7 (median: 3, mean: 3.05). Figure 1.3.4 illustrates the relationship between counts in cores at the two time points. Mvc in cores at diagnosis (mvc1) ranged from 10 to 22 (mean: 16.4, median: 16.5) and mvc in cores at surgery (mvc2) ranged from 13 to 21 (mean: 17.4, median: 18). No correlation was found between the groups (r=-0.38). There was no significant difference in mvc between the two groups (p=0.481). Mvc was higher in later cores in 5 of 10 tumours (50%), higher in initial biopsies in 4 tumours (40%) and the same in one (10%).

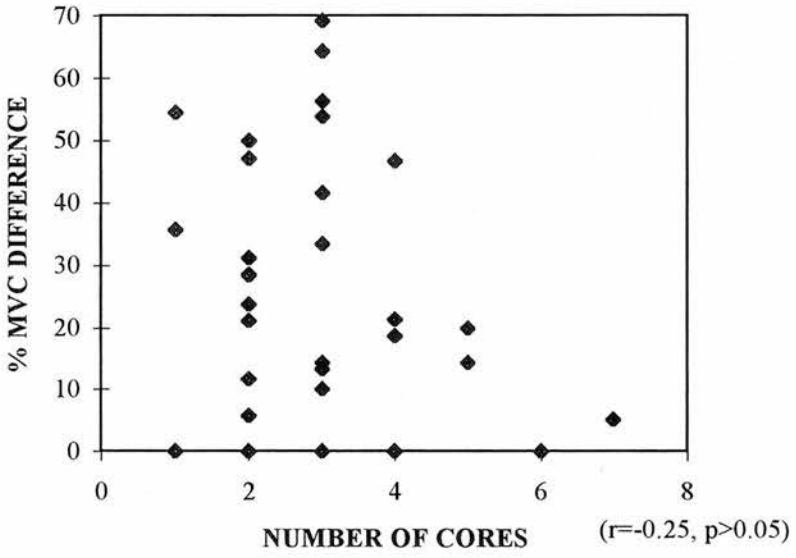
Figure 1.3.4: Relationship between mvc of cores taken at separate time points.



Correlation between number of cores performed and difference in mvc between cores and tumour section.

Analysis was performed to determine whether there was a relationship between number of cores performed and the difference in mvc between cores and tumour sections. In 37 cases, cores and tumour section were available for comparison. The number of cores performed ranged from 1 to 7 (median: 3, mean: 3.13). The difference in mvc was expressed as a percentage of total mvc count in the tumour section, and its relationship with number of cores is illustrated on Figure 1.3.5. No significant correlation was identified ($r=-0.25$, $p>0.05$).

Figure 1.3.5: % difference in mvc between two tumour samples versus number of cores performed.



1.4. Discussion:

The present study has attempted to assess the variation in assessment of angiogenesis which might be caused by (i) inherent variation in the method, (ii) tumour heterogeneity and (iii) previous surgical manipulation of tumour.

Variation in microvessel counts (mvc) between the same observers performed at different times was 11% in this study, with a highly significant correlation between the two sets of counts ($r=0.84$, $p<0.001$). Interobserver variation was 15%, which is comparable to another study which addressed the same issue (Martin *et al*, 1997). The issue of observer variation has been raised in several studies as a possible reason why angiogenesis assessment has failed to provide useful prognostic information in some series of primary breast cancers (Axelsson *et al*, 1995; Van Hoef *et al*, 1993). In both studies, mvc was used as a categorised rather than a continuous variable (Axelsson *et al*, 1995; Van Hoef *et al*, 1993), which may result in loss of useful prognostic information (Vermeulen *et al*, 1996). Another study addressing the issue of observer variation found that mvc provided independent prognostic information in a series of breast tumours when performed by experienced observers but not by the least experienced observer (Vermeulen *et al*, 1997b). The authors therefore recommended that a period of training with an experienced observer is advisable (Vermeulen *et al*, 1997b). In the present study, counts were performed by two observers, a pathologist and a surgeon who had undertaken a period of training with an experienced observer prior to embarking on the study. Other methods have been suggested to improve objectivity of counting such as using a Chalkley eyepiece graticule, as in the present study, or computerised image analysis (Fox *et al*, 1995a). In a recently published study comparing interobserver variability using different counting methods, use of the Chalkley grid yielded low variability and was found to be more effective in reducing variation than selection of the same hot spot by different observers (Hansen *et al*, 1998). Reproducibility was further optimized by the use of a conference microscope, thereby eliminating subjectivity in hot spot selection and field sampling (Hansen *et al*, 1998). The low degree of interobserver variability observed in the present study would therefore be compatible with the use of the Chalkley grid by two observers using a conference microscope.

Variation in staining due to use of two endothelial cell antibodies has been assessed. Studies investigating the role of mvc as a prognostic indicator in breast cancer have used both CD31

and FVIII antibodies, in addition to another antibody, CD34 (Van Hoef *et al*, 1993; Weidner *et al*, 1992). Several studies have compared use of different antibodies. In an early study comparing CD34, CD31 and Factor VIII, CD31 antibody was found to be the most sensitive, consistently staining more vessels than either antibody to CD34 or Factor VIII (Horak *et al*, 1992). A later study found CD34 to be most strongly and consistently expressed with little background staining and no staining of other cells (Martin *et al*, 1997). Antibody to Factor VIII stains consistently, but has the disadvantage of staining lymphatic in addition to vascular endothelium (Martin *et al*, 1997; Vermeulen *et al*, 1996). CD31, the most sensitive marker of endothelial cells, stains microvessels as well as larger vessels but has the disadvantage of frequent antigen loss due to certain fixatives (Vermeulen *et al*, 1996). It was because of these potential problems that sections were stained with both antibodies to CD31 and Factor VIII. There was a highly significant correlation between the two groups of counts ($r=0.4$, $p<0.0001$) in both core biopsy and tumour sections.

Tumour heterogeneity in vasculature has been investigated in the present study by comparing microvessel counts in core biopsies and tumour sections taken simultaneously. Whilst the range of counts was similar in the two groups, correlation between counts failed to reach statistical significance ($r=0.44$, $p=0.09$), with vessel counts in tumour sections tending to be higher than core biopsies. This may be related to the methodology used in counting. Heterogeneity of tumour vasculature between different areas of the same tumour is well-recognised (Van Hoef *et al*, 1993; Weidner *et al*, 1992) and may be overcome to an extent by selection of three vascular 'hot spots'. Selection of such 'hot spots' remains the most subjective aspect of microvessel counting, although a recent study suggested that the use of a Chalkley grid was more important at improving objectivity than selection of the same hot spot by different observers (Hansen *et al*, 1998). The reason given for this was that, even within the same hot spot, different high power fields were likely to be selected by different observers (Hansen *et al*, 1998). Alternatives to the original method proposed by Weidner have been suggested, which may be of particular benefit to the less experienced observer. Firstly it has been suggested that counts be performed in ten fields of high vascularity, taking the highest count as the mvc (Martin *et al*, 1997). An alternative method would be to perform photomicrographs of the most intensely vascular areas and for individual observers to count from the photographs (Vermeulen *et al*, 1997b). Core biopsies contain a small section of total tumour and are therefore less likely to include vascular 'hot spots' than a tumour section. This has been borne out in results of the present study, and is demonstrated in Figure 1.3.1, which illustrated the difference in section obtained from the

same tumour by core biopsy (a) and tumour cross-section (b), both taken at the time of surgery. Counts in core biopsies were lower than tumour sections in 56% of tumours studied. Another study which addressed the role of core biopsies found a similar lack of correlation in mvc of cores and tumour sections, but found mvc to be higher in core biopsies than tumour sections in 61.2% of cases (Jacobs *et al*, 1998).

Problems with tumour heterogeneity are not unique to vascular assessments, but have been described for several other parameters in breast cancer. Significant variations in mitotic activity index assessed on haematoxylin and eosin sections (Jannink *et al*, 1996) and DNA cell cycle variables (Bergers *et al*, 1996) were found on assessment of multiple tumour sections. Conversely, levels of MIB-1 and oestrogen receptor expression were found to alter little in assessments of serial tumour sections (Jensen & Ladekarl, 1995).

The issue of the accuracy of core biopsies in providing prognostic information is important as such specimens are increasingly used in diagnosis and may provide the only pre-treatment tissue available. Other studies have addressed similar issues for markers other than angiogenesis and reproducible results have been obtained for determinations of DNA ploidy, bcl-2, oestrogen receptor, c-erbB-2 and p53, which are dichotomously scored variables (Daidone *et al*, 1991; Jacobs *et al*, 1998). Jacobs et al failed to produce reproducible results for microvessel counts and accuracy was not improved by increasing tumour area available for assessment by performing more core biopsies (Jacobs *et al*, 1998). This is in keeping with results from the present study, in which, whilst there is a suggestion from Figure 1.3.5 that difference in mvc may be reduced with an increasing number of the core biopsies, this correlation failed to reach statistical significance.

Another possible reason for unreliablility of sequential core biopsies in tumour vascular assessment is the likely induction of angiogenesis following the initial biopsy as part of the normal process of wound healing (Folkman, 1971). Comparison of microvessel counts in core biopsies and subsequent core biopsies with no intervening treatment failed to show any correlation. In particular, it is worth noting that counts were found to be higher in the later tumour samples in 65% of cases, which suggests that angiogenesis may well have been induced following the early biopsy. However, this trend failed to reach statistical significance.

Various practical issues regarding the methodology of tumour vascularity assessment by microvessel counts have been investigated. Intra- and inter-observer variations in counts were 11 and 15% respectively, which were similar to those described in other studies. Tumour heterogeneity is a problem when adopting this method in small tumour biopsies, such as core biopsies, which may not contain vascular 'hot spots'. In addition, angiogenesis takes place in conditions other than tumour growth, such as wound healing, and is therefore likely to be stimulated by surgical manipulations of the tumour, affecting subsequent assessments. Results of the present study suggest that microvessel counts on core biopsy specimens are unlikely precisely to represent overall vascularity of the individual tumour. In determining the role of assessment of sequential core biopsies during primary systemic therapy, the size of the effect of therapy will be important. If tamoxifen exerts a dramatic effect on tumour vascularity this may be apparent in core biopsies despite the shortcomings of the technique demonstrated in this study. Against this background a series of studies aimed at determining the effect of tamoxifen on breast tumour vascularity were undertaken.

2. Changes in tumour vascularity following primary tamoxifen treatment and correlation with response.

Having investigated the method of performing microvessel counts in terms of observer variation and reproducibility in different breast cancer specimens, the focus of the remaining chapters is the effect of tamoxifen on breast tumour vascularity. The aim of the study outlined in this chapter was to determine whether changes in tumour vascularity take place following primary treatment with tamoxifen in relation to response. A study of patients treated with neoadjuvant tamoxifen has been undertaken, in which tumour tissue was available before and after treatment, allowing comparison of tumour vascularity in association with response at the two time points.

Seventy-two post-menopausal women with large (>3cm), operable or locally advanced (ipsilateral lymph node or skin involvement) breast cancers which were oestrogen receptor (ER)-positive (>20fmol/mg cytosolic protein) were recruited into this retrospective study. Patients were treated with tamoxifen (20mgs daily) for a period of between three and thirty months, following a wedge biopsy for determination of ER status and prior to definitive surgery. Tumour response to tamoxifen was determined by monthly clinical, ultrasound and mammographic assessments of tumour volume. This study design allowed comparison of tumour vascularity before and after treatment with tamoxifen and correlation with response.

Fifty-seven of the seventy-two patients (79%) were treated with tamoxifen for between three and six months. Fifteen patients continued to take tamoxifen for between seven and thirty months. This group of patients had locally advanced disease, with skin or axillary lymph node involvement. During early treatment with tamoxifen, tumour growth was controlled, but escaped control subsequently, therefore requiring later surgery. The response patterns of these tumours were, therefore, complex and will be considered separately.

In this chapter results of 57 patients treated with tamoxifen for between three and six months will be detailed first, including tumour response data, assessments of tumour vascularity by microvessel counting and correlation between the two. Raw data are detailed in the Appendix. In addition, similar analyses were performed on twenty-two patients who were treated with the aromatase inhibitor, letrozole, allowing comparison of effects of the two drugs.

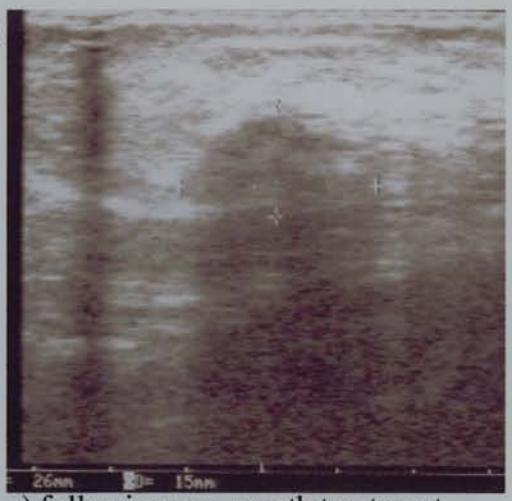
2.1. Clinical assessment of tumour response to tamoxifen.

Patients were assessed following four, eight and twelve weeks' treatment with tamoxifen. Tumour volumes were assessed by caliper measurements and ultrasound measurements of anteroposterior and transverse diameters and depth were taken. Patients underwent mammography before and after treatment. In 30 patients, details of ultrasound assessments (US) were available and these were used to determine response. In 22 patients, insufficient ultrasound data were available but there was clear clinical evidence of response (CLIN). In five patients, mammographic information alone was available (MAMM.).

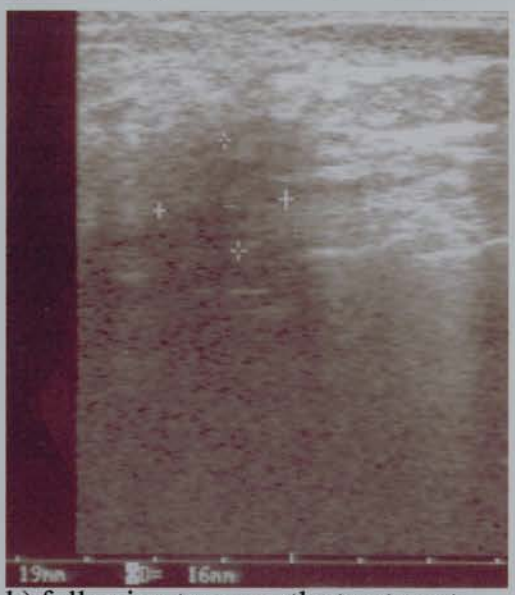
A responding tumour was defined as one in which there was a greater than 25% reduction in tumour volume at the end of three months. Ultrasound measurement of tumour volume was the preferred method of assessment, but when unavailable, response was defined according to changes in clinical or mammographic measurements. Sequential ultrasound assessments of a responding tumour during the course of treatment are illustrated on Figure 2.1.1, and pre- and post-treatment mammograms are illustrated on Figure 2.1.2.

Details of changes in tumour volume relative to original volume for each assessable patient are shown on Table 2.1.1. Mean serial reductions in tumour volume in non-responding and responding tumours are illustrated on Figure 2.1.3. Mean reduction in volume in non-responding tumours at completion of treatment was 6%, and in responding tumours was 64%. Numbers of patients with responding and non-responding tumours are shown on Table 2.1.2 and demonstrates an overall response rate of 75%.

Fig 2.1.1. Serial ultrasound assessments of tumour volume demonstrating a reduction in tumour volume with treatment.



a) following one month treatment



b) following two months treatment

c) following three months treatment

Fig 2.1.2. Mammographic evidence of tumour response

a) Tumour volume before treatment

b) Reduced tumour volume following three months of tamoxifen treatment.

Table 2.1.1: Changes in relative tumour volume during treatment with tamoxifen.

Tumour volumes are expressed as % original volume, non-responders shown in **bold** type.

Tumour vol. 1=volume following 1 month of treatment

Tumour vol. 2=volume following two months of treatment

Tumour vol. 3=volume following three months of treatment

Mam. Resp.=response seen on mammography.

NA=results not available

a) Non-responding tumours:

Patient No.	Original vol	Tumour vol 1	Tumour vol 2	Tumour vol 3	Mam. Resp..	Assessment.
480425W	100	100	70	97	static	US
495639K	100	100	100	100	static	US
558680X	100	100	70	100	reduction	US
495980E	100	100	100	100	NA	CLIN
494064R	100	100	100	100	NA	CLIN
545602E	100	100	91	81	static	CLIN
582565W	100	79	100	100	static	US
528456W	100	100	100	82	reduction	US
572868X	100	100	87	87	increase	US
596319W	100	100	100	79	NA	US
522679X	100	100	100	100	NA	CLIN
502246A	100	100	100	100	NA	CLIN
524732W	100	100	100	100	increase	MAMM.
524111X	100	100	88	96	reduction	US

b) Responding tumours:

Patient No.	Original vol	Tumour vol 1	Tumour vol 2	Tumour vol 3	Mam. Resp..	Assessment.
268300H	100	35	24	8	NA	US
110258V	100	100	100	59	NA	US
528171K	100	100	73	73	NA	US
552168H	100	100	79	35	reduction	US
52346X	100	100	72	52	reduction	MAMM.
560577L	100	100	53	27	NA	US
513260L	100	24	12	12	NA	CLIN
527028L	100	61	18	9	no lump	US
44955M	100	100	54	reduction	NA	CLIN
395041K	100	100	44	20	NA	US
350429H	100	100	73	20	NA	US
545123B	100	100	77	9	NA	US
520042V	100	100	57	57	progression	CLIN

Patient No.	Original vol	Tumour vol 1	Tumour vol 2	Tumour vol 3	Mam. Resp..	Assessment.
81364H	100	100	72	47	decrease	MAMM.
511159M	100	100	71	55	reduction	CLIN
500371K	100	81	73	22	NA	CLIN
372859M	100	59	69	55	reduction	US
529249W	100	100	38	33	no lump.	CLIN
376029R	100	61	61	60	NA	US
499870K	100	83	71	71	NA	US
520767V	100	100	62	51	NA	CLIN
498214W	100	91	51	13	reduction	US
498258R	100	100	100	68	NA	US
559968K	100	41	23	17	NA	US
510657M	100	70	45	28	reduction	CLIN
450908A	100	100	45	36	reduction	US
155271L	100	100	77	70	NA	US
519014W	100	100	100	45	no change	CLIN
499532M	100	100	47	35	reduction	US
564461M	100	36	36	36	reduction	CLIN
515126A	100	100	89	68	NA	CLIN
529146X	100	83	38	15	reduction	MAMM.
458317M	100	100	24	27	reduction	US
548395L	100	33	16	16	increase	US
509362K	100	NA			NA	CLIN
503527E	100	NA			NA	CLIN
526825K	100	NA			reduction	MAMM.
488627M	100	38	2	2	NA	US
535168M	100	NA			NA	CLIN
532363A	100	100	58	32	reduction	CLIN
527259E	100	52	44	39	NA	CLIN
527857A	100	NA			NA	CLIN
588347R	100	100	100	51	reduction	US

Figure 2.1.3: Mean serial changes in tumour volume in non-responding (n=14) and responding (n=43) tumours.
Standard error bars shown.

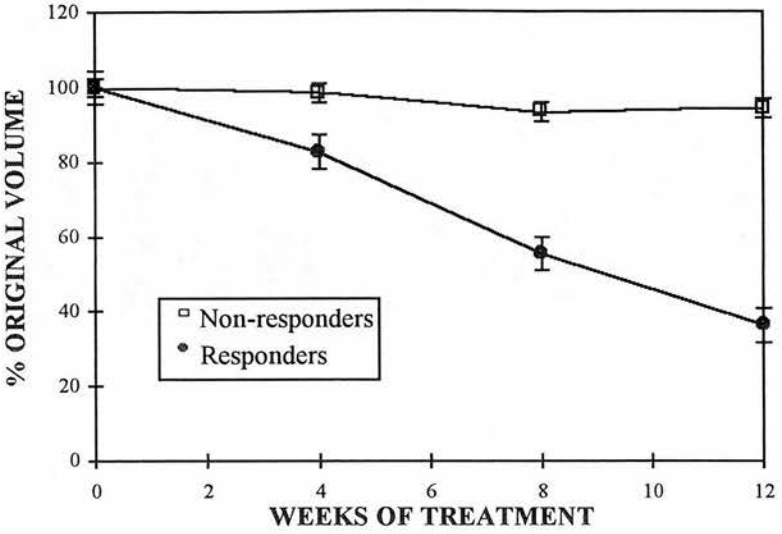


Table 2.1.2: Number of patients defined by response to tamoxifen:

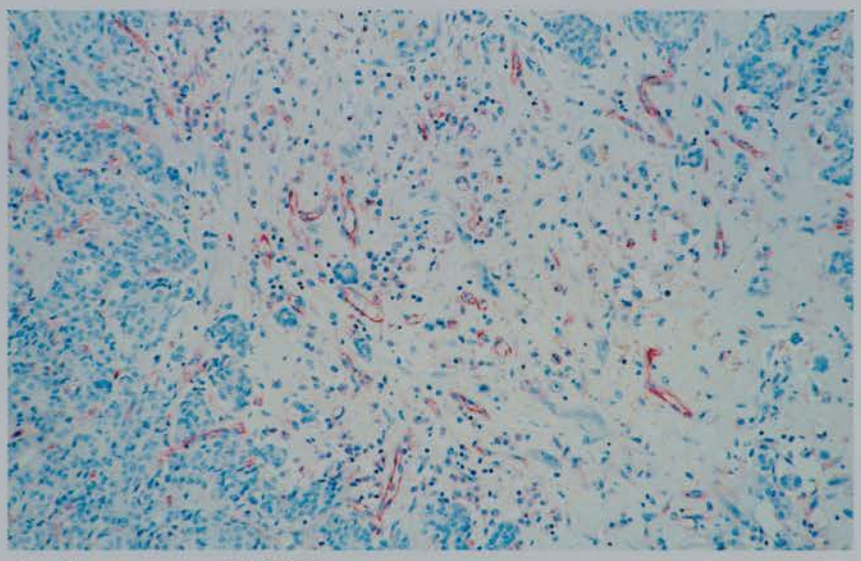
RESPONDERS	43 (75%)
NON-RESPONDERS	14 (25%)
TOTAL	57 (100%)

2.2 Microvessel counts following staining with antibody to Factor VIII.

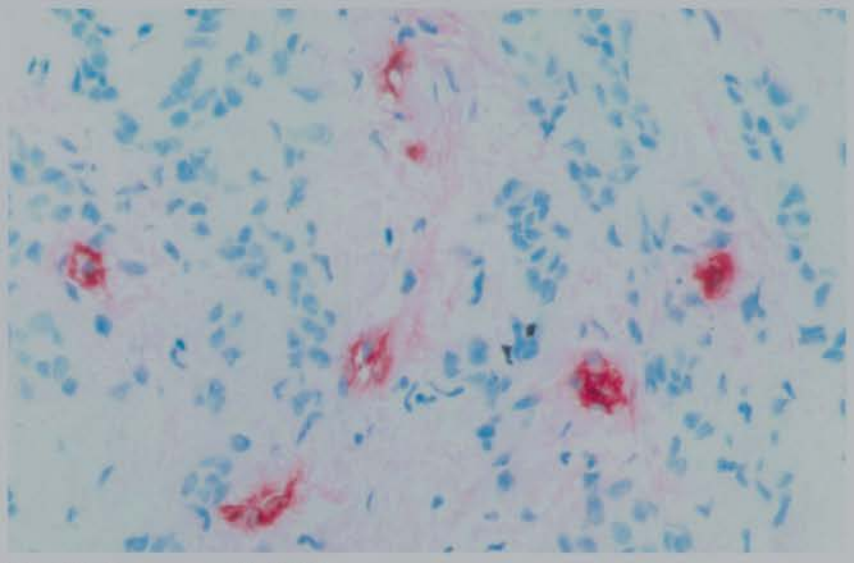
Assessments of tumour vascularity were made by performing microvessel counts in three areas of high vascularity in all tumours before and after treatment with tamoxifen for a period of between three and six months. The microvessel count of each tumour section was defined as the total of the three counts. Endothelial cell staining was performed using immunohistochemical techniques as described in the Methods section. Sequential tumour sections were stained with antibody to Factor VIII (Von Willebrand factor) and antibody to CD31. Microvessel counts obtained following staining with antibody to Factor VIII will be considered first.

Figure 2.2.1 illustrates an example of a tumour section following staining with antibody to Factor VIII.

Fig 2.2.1. Tumour section following endothelial cell staining with antibody to Factor VIII. Vessels are highlighted in red with haematoxylin counterstaining.



a) at low power (X 100)



b) at high power (X 250)

Comparison of pre- and post-treatment mvc in all tumours is illustrated on Figure 2.2.2, demonstrating no significant difference in counts with treatment. Range of mvc in all tumours before treatment was 10-35 (median: 17, mean: 17.3) and after treatment was 7-32 (median: 16, mean: 16.3). Data are summarised on Table 2.2.1.

Figure 2.2.2: Microvessel counts before and after tamoxifen treatment in all patients.

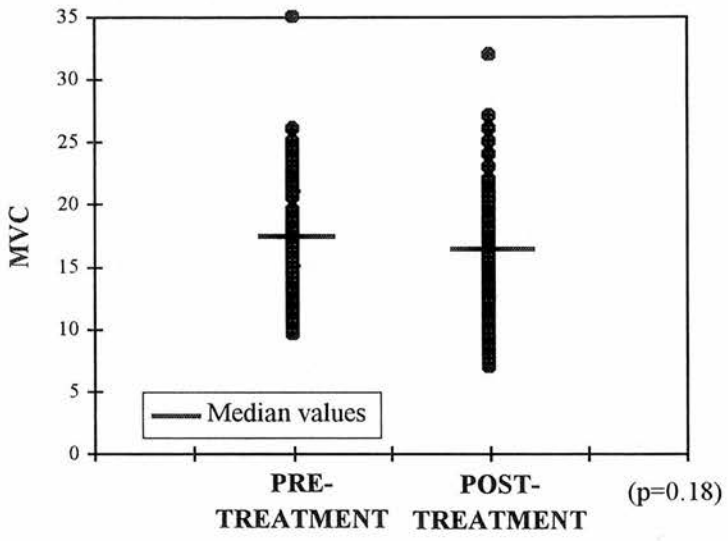


Table 2.2.1: Summary of pre- and post-treatment mvc in all tumours:

	Pre-treatment mvc	Post-treatment mvc
Range	10-35	7-32
Mean	17.3	16.3
Median	17	16
Standard deviation	4.77	5.42
Standard error	0.63	0.72

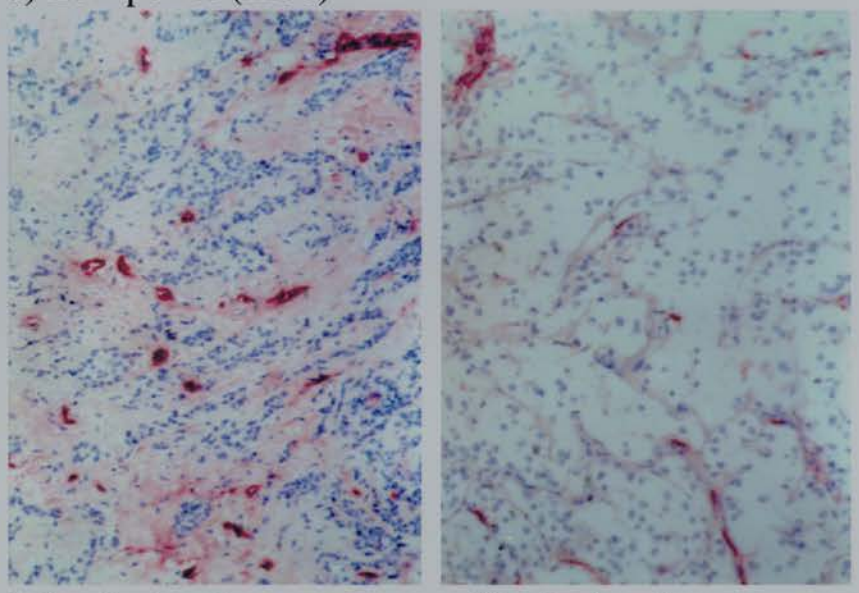
Paired Wilcoxon signed rank test: p=0.18

Microvessel counts and response.

Examples of changes in microvessel counts in individual tumours following treatment are illustrated on Figures 2.2.3 and 4, demonstrating a reduction in mvc following treatment (Figure 2.2.3) and an increased mvc (Figure 2.2.4). Such changes were correlated with response.

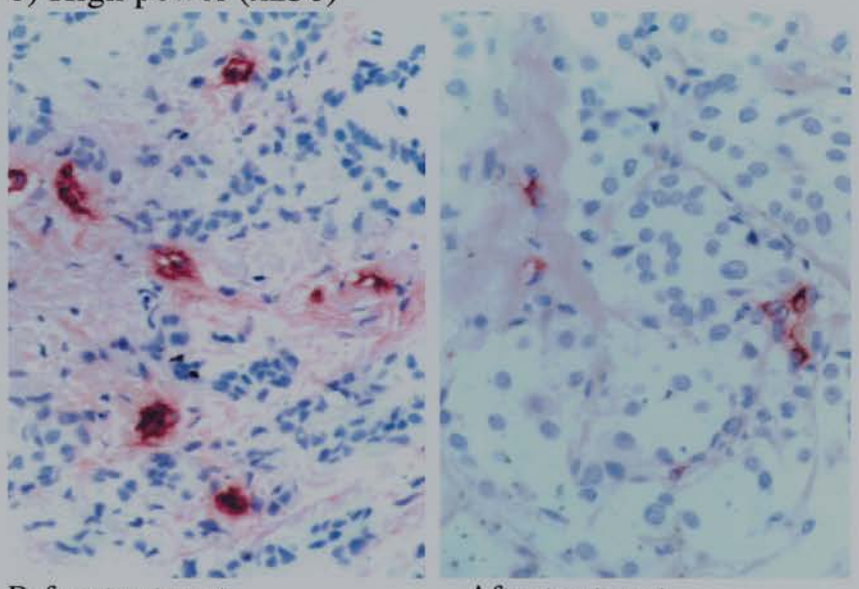
Fig 2.2.3 Reduction in microvessel density following tamoxifen treatment.

a) Low power (x100)



Before treatment | After treatment

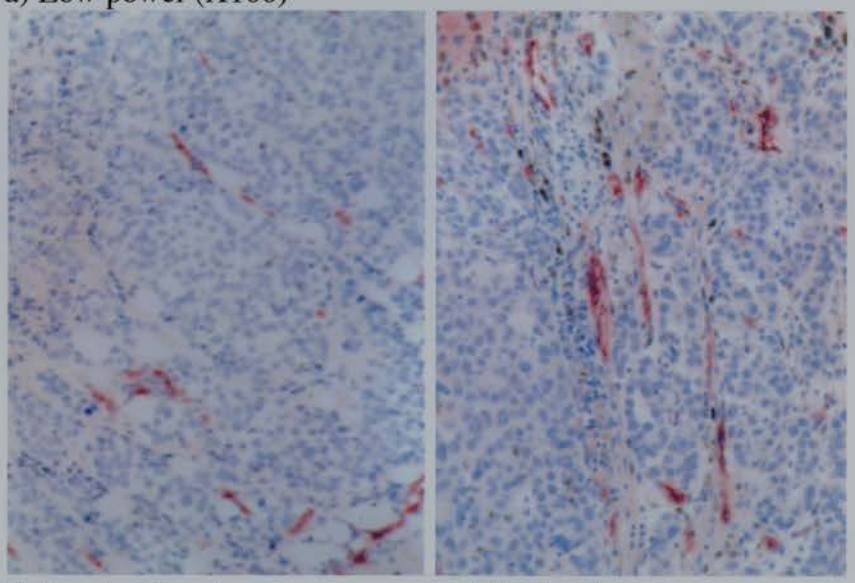
b) High power (x250)



Before treatment | After treatment

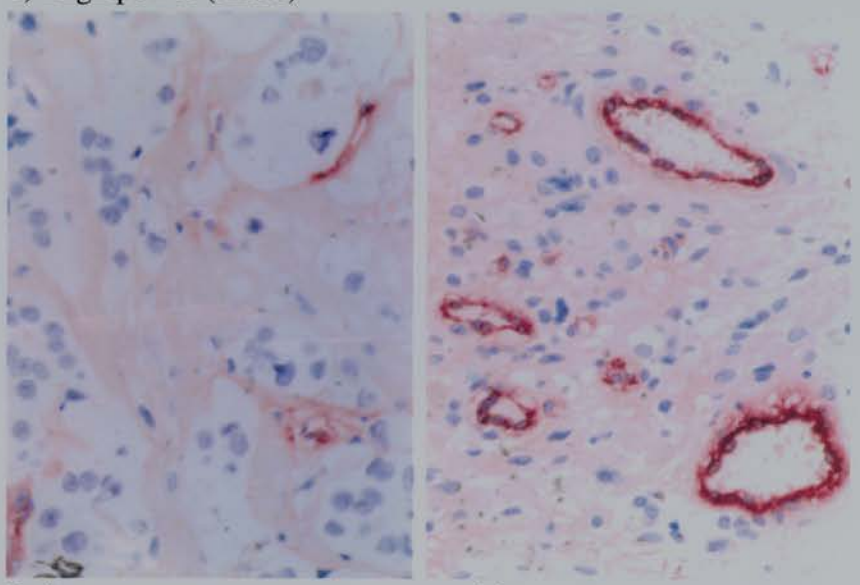
Fig 2.2.4. Increase in microvessel density following tamoxifen treatment.

a) Low power (X100)



Before treatment After treatment

b) High power (X250)



Before treatment After treatment

Pre-treatment mvc: No difference in pre-treatment mvc between non-responding and responding tumours (p=0.79) was observed, as illustrated in Figure 2.2.5. The range of pre-treatment mvc in non-responding tumours was 12-21 (mean: 16.6, median: 17) and in responding tumours was 10-35 (mean: 17.5, median: 17).

Figure 2.2.5: Comparison of pre-treatment mvc in non-responding (n=14) and responding (n=43) tumours.

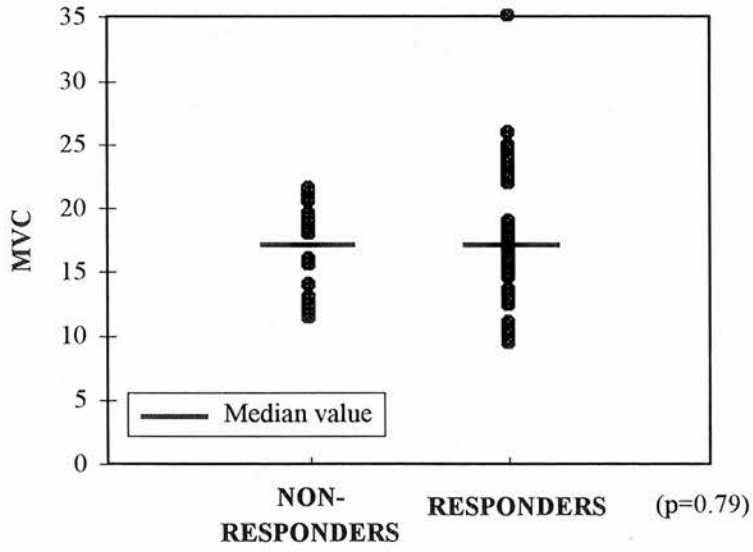


Table 2.2.2: Summary of pre-treatment mvc data:

	Pre-treatment mvc in non-responding tumours	Pre-treatment mvc in responding tumours
Range	12-21	10-35
Median	17	17
Mean	16.64	17.54
Standard deviation	3.52	5.13
Standard error	0.94	0.78

Mann-Whitney test: p=0.79

Post-treatment mvc: A significant difference in post-treatment mvc between non-responders (range: 15-27, mean: 19.9, median: 19) and responders (range: 7-32, mean: 15.1, median: 15) was identified (p=0.0027) and is illustrated on Figure 2.2.6.

Figure 2.2.6: Comparison of post-treatment mvc in non-responding and responding tumours.

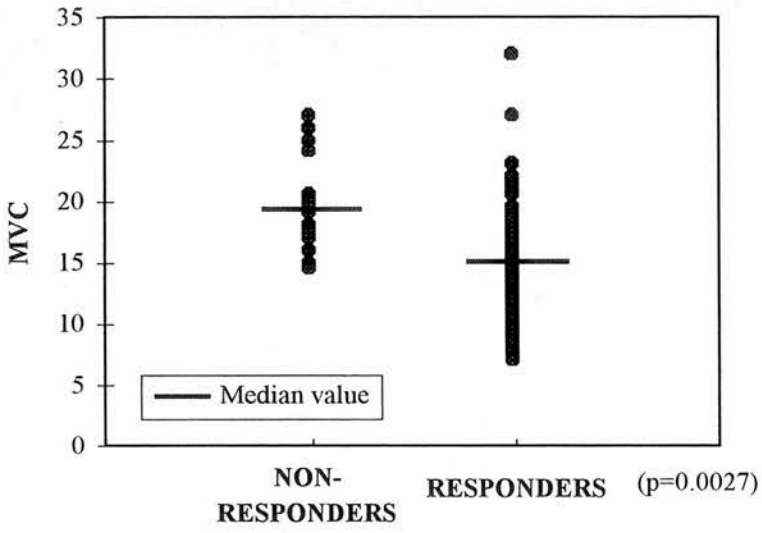


Table 2.2.3: Summary of post-treatment mvc data:

	Post-treatment mvc in non-responding tumours	Post-treatment mvc in responding tumours
Range	15-27	7-32
Median	19	15
Mean	19.86	15.14
Standard deviation	4.07	5.33
Standard error	1.089	0.814

Mann-Whitney test: p=0.0027

Changes in microvessel count in responding and non-responding tumours following treatment with tamoxifen.

Responding tumours: There was a significant reduction in mvc in responding tumours following treatment, as illustrated on Figure 2.2.7 (p=0.0064). The range of mvc before treatment was 10-35 (mean: 17.5, median: 17) and after treatment was 7-32 (mean: 15.1, median: 15).

Figure 2.2.7: Comparison of pre- and post-treatment mvc in responding tumours.

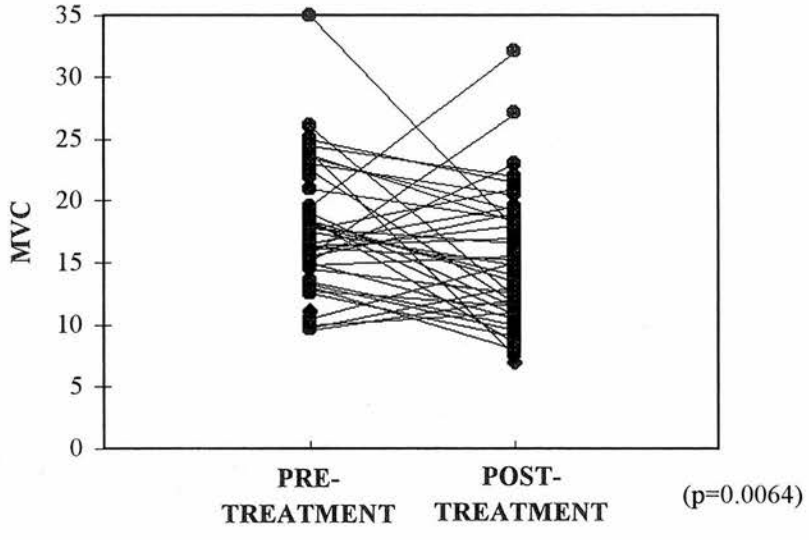


Table 2.2.4: Summary of tumour vascularity data for responding tumours:

	Pre-treatment mvc	Post-treatment mvc
Range	10-35	7-32
Median	17	15
Mean	17.54	15.14
Standard deviation	5.13	5.33
Standard error	0.78	0.81

Paired Wilcoxon signed rank test: p=0.0064

Non-responding tumours: There was a significant increase in mvc in non-responding tumours following treatment, as illustrated on Figure 2.2.8 (p=0.036). The range of mvc before treatment was 12-21 (mean: 16.6, median: 17) and after treatment was 15-27 (mean: 19.9, median: 19).

Figure 2.2.8: Comparison of pre- and post-treatment mvc in non-responding tumours.

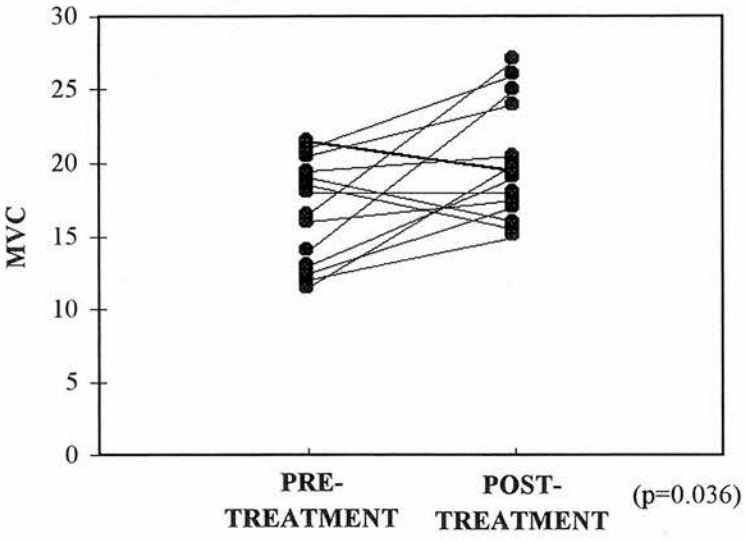


Table 2.2.5: Summary of tumour vascularity data for non-responding tumours:

	Pre-treatment mvc	Post-treatment mvc
Range	12-21	15-27
Median	17	19
Mean	16.64	19.86
Standard deviation	3.52	4.07
Standard error	0.94	1.089

Paired Wilcoxon signed ranked test: p=0.036

Changes in microvessel counts with response to primary tamoxifen treatment.

The numbers of patients in whom mvc increased, decreased or remained static are shown on Table 2.2.6. In order to be defined as a change in mvc, the difference between pre- and post-treatment counts had to be greater than 10% of post-treatment count, corresponding to the degree of interobserver variation, as described previously. A clear trend in change of microvessel count was identified, with an increase in mvc in the majority of non-responding tumours and a decrease in the majority of responding tumours. It can be observed, however, that there is a subset of responding tumours (23%) in which mvc increased, and of non-responding tumours (21.6%) in which mvc decreased with treatment.

Table 2.2.6: Correlation of change in microvessel counts with treatment response:

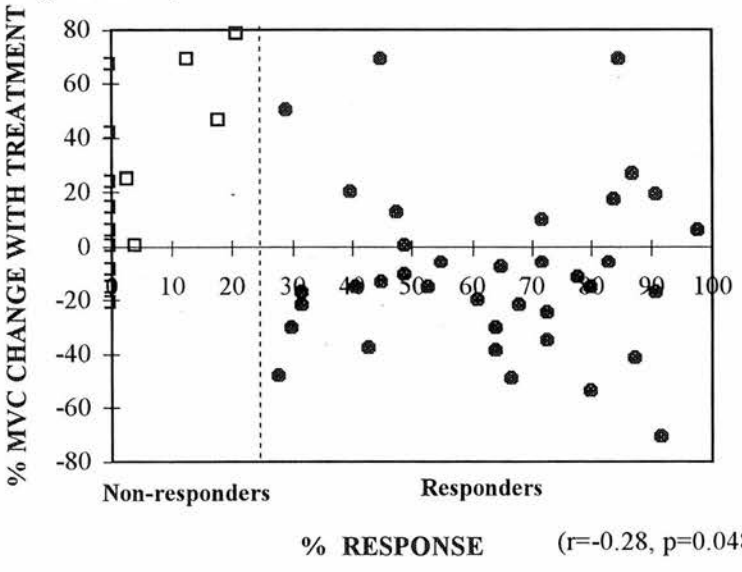
	Increased MVC	**MVC static**	**Decreased MVC**	**TOTAL**
Non-responders	8 (57%)	3 (21.5%)	3 (21.5%)	**14**
Responders	10 (23%)	6 (14%)	27 (63%)	**43**

Chi-squared test for trend: p=0.0062

Percentage reduction in tumour volume and change in microvessel count.

Results detailed in this chapter have demonstrated a significant reduction in mvc following staining with antibody to Factor VIII in responding tumours and an increase in non-responding tumours. The relationship between extent of tumour response to treatment and degree of change in vascularity is now addressed. Tumour response is expressed as a percentage reduction in original tumour volume determined by ultrasound or clinical assessments as described previously. Five patients in whom response was determined by mammographic changes alone were excluded from this analysis. The relationship is shown on Figure 4.11 and demonstrates a significant association between degree of response to treatment and % change in mvc (r=-0.28, p=0.048).

Figure 2.2.9: Relationship between % mvc change and degree of response to treatment.



2.3 Microvessel counts following staining with antibody to CD31.

Sections of all tumours before and after treatment with tamoxifen were stained with antibody to CD31 in addition to Factor VIII antibody. Some sections failed to stain positively for antibody to CD31 despite repeated attempts. In sections that were negative, vessels were visible on the H and E slide, but failed to stain. Staining was unsuccessful in both pre- and post-treatment sections of 7 tumours, in pre-treatment sections only of 5 tumours and 8 post-treatment tumours. Results of CD31 staining are detailed in the following section: in sections that did not stain mvc was taken as zero. Failure of staining may be due to methodological problems or may occur as a result of tamoxifen treatment. The possible explanations will be discussed at the end of the chapter.

Ranges of microvessel count following CD31 staining in all tumours before and after treatment are illustrated in Figure 2.3.1. The range of mvc before treatment was 0-35 (mean: 14.7, median: 16) and after treatment was 0-28 (mean: 11.9, median: 12). There was a significant reduction in mvc following treatment in the total study population ($p=0.02$). The data are summarised on Table 2.3.1.

Figure 2.3.1: Microvessel counts before and after tamoxifen treatment in all patients.

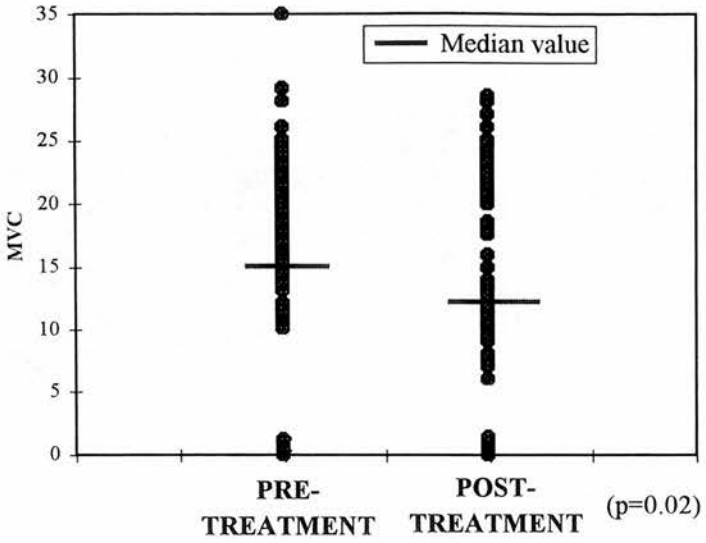


Table 2.3.1: Summary of pre- and post-treatment mvc in all tumours:

	Pre-treatment mvc	Post-treatment mvc
Range	0-35	0-28
Median	16	12
Mean	14.7	11.9
Standard deviation	8.99	9.03
Standard error	1.19	1.20

Paired Wilcoxon signed rank test: p=0.02

Microvessel counts and response.

Pre-treatment mvc: Pre-treatment mvc in non-responding and responding tumours are compared in Figure 2.3.2, demonstrating no significant difference between the two groups. Range of mvc in non-responding tumours was 0-29 (median: 15, mean: 12.6), and in responding tumours 0-35 (median: 17, mean: 15.4). Data are summarised on Table 2.3.2.

Figure 2.3.2: Comparison of pre-treatment mvc in non-responding (n=14) and responding (n=43) tumours.

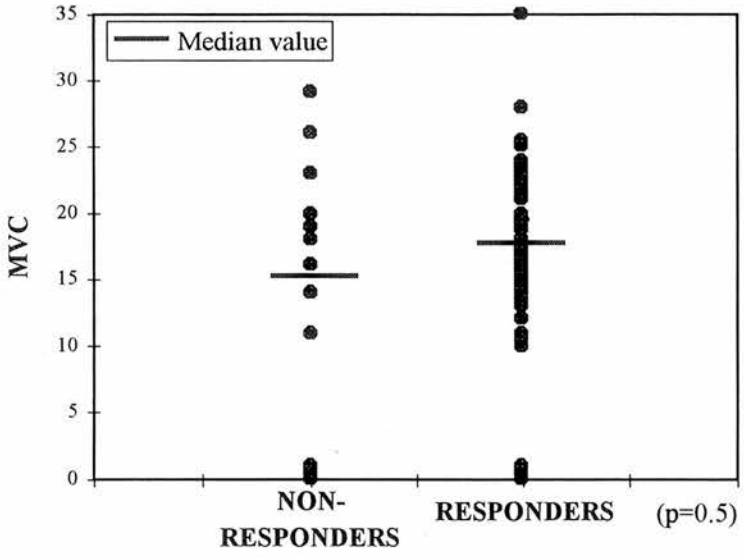


Table 2.3.2: Summary of pre-treatment mvc data:

	Pre-treatment mvc in non-responding tumours	Pre-treatment mvc in responding tumours
Range	0-29	0-35
Median	15	17
Mean	12.6	15.4
Standard deviation	10.7	8.39
Standard error	2.86	1.28

Mann-Whitney test: p=0.5

Post-treatment mvc: Post-treatment mvc in non-responding and responding tumours are compared in Figure 2.3.3, demonstrating no significant difference between the two groups (p=0.75). Range of mvc in non-responding tumours was 0-28 (median: 14.5, mean: 11.6), and in responding tumours 0-28 (median: 12, mean: 11.6). Data are summarised on Table 2.3.3.

Figure 2.3.3: Comparison of post-treatment mvc in non-responding and responding tumours.

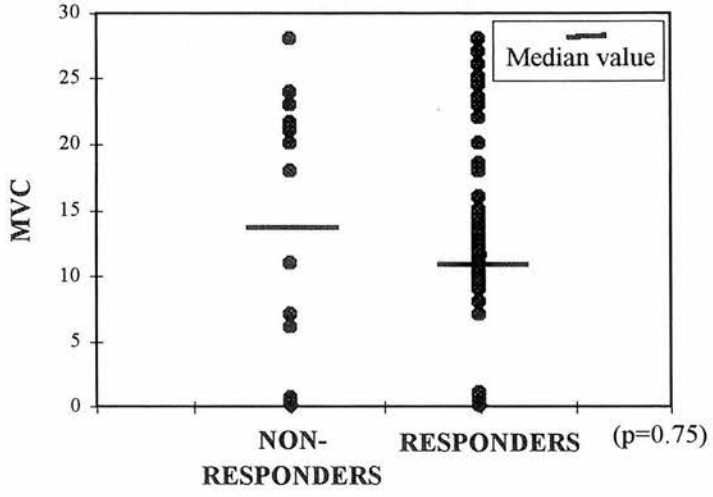


Table 2.3.3: Summary of post-treatment mvc data:

	Post-treatment mvc in non-responding tumours	Post-treatment mvc in responding tumours
Range	0-28	0-28
Median	14.5	12
Mean	12.8	11.6
Standard deviation	10.4	8.7
Standard error	2.79	1-32

Mann-Whitney test: p=0.75

Changes in microvessel count in responding and non-responding tumours following treatment with tamoxifen.

Responding tumours: There was a significant reduction in mvc in responding tumours following treatment with tamoxifen, as illustrated in Figure 2.3.4 (p=0.02). Range of mvc before treatment was 0-35 (median: 17, mean: 15.4) and after treatment was 0-28 (median: 12, mean: 11.6). Data are summarised in Table 2.3.4.

Figure 2.3.4: Comparison of pre- and post-treatment mvc in responding tumours.

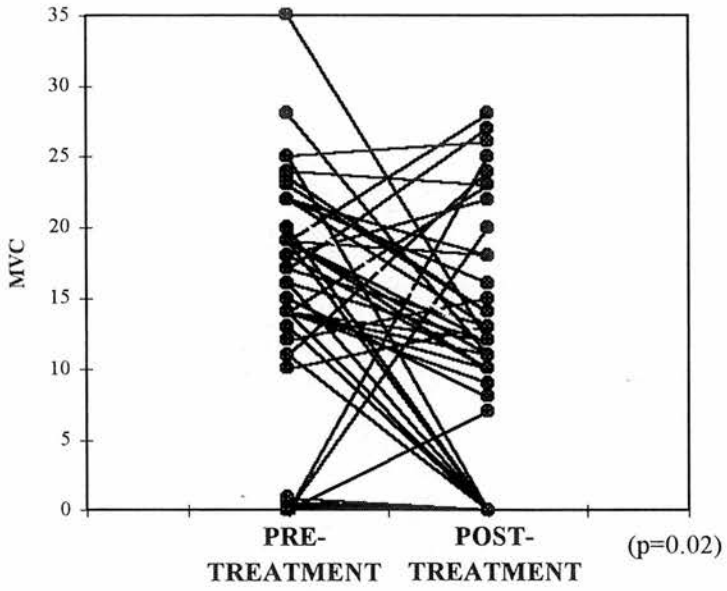


Table 2.3.4: Summary of tumour vascularity data for responding tumours:

	Pre-treatment mvc	Post-treatment mvc
Range	0-35	0-28
Median	17	12
Mean	15.4	11.6
Standard deviation	8.39	8.65
Standard error	1.28	1.32

Paired Wilcoxon signed rank test: p=0.02

Non-responding tumours: No significant change in mvc with treatment was observed in non-responding tumours, as illustrated on Figure 2.3.5. Pre-treatment mvc ranged from 0-29 (median: 15, mean: 12.6) and post-treatment mvc ranged from 0-28 (median: 14.5, mean: 12.8). Data are summarised on Table 2.3.5.

Figure 2.3.5: Comparison of pre- and post-treatment mvc in non-responding tumours.

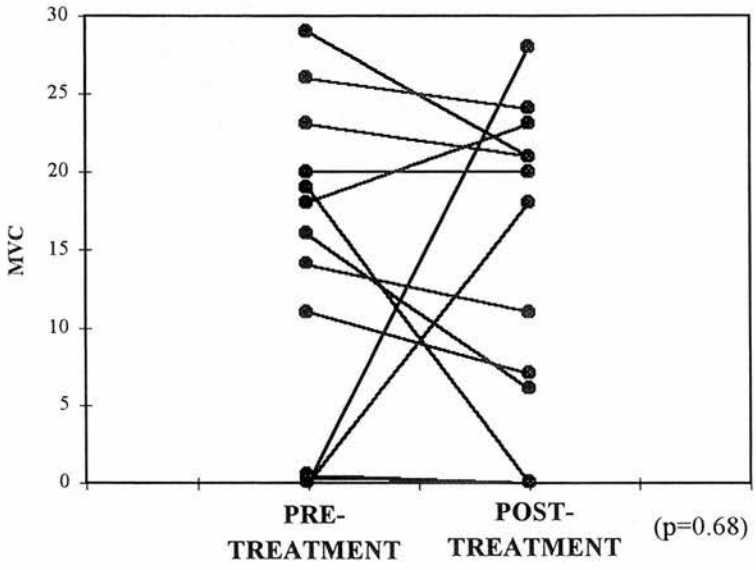


Table 2.3.5: Summary of tumour vascularity data for non-responding tumours:

	Pre-treatment mvc	Post-treatment mvc
Range	0-29	0-28
Median	15	14.5
Mean	12.6	12.8
Standard deviation	10.7	10.4
Standard error	2.86	2.79

Paired Wilcoxon signed rank test: p=0.68

Changes in microvessel counts with response to primary tamoxifen treatment.

Numbers of patients in whom mvc increased, decreased or remained static are shown on Table 2.3.6. Of 14 non-responding tumours, mvc reduced in seven, remained static in four and increased in three. These findings differ from the trend observed following staining with antibody to Factor VIII. A similar trend to that observed following Factor VIII staining was observed in responding tumours: mvc reduced in 26 tumours, remained static in seven and increased in ten.

Table 2.3.6: Correlation of change in microvessel counts with treatment response:

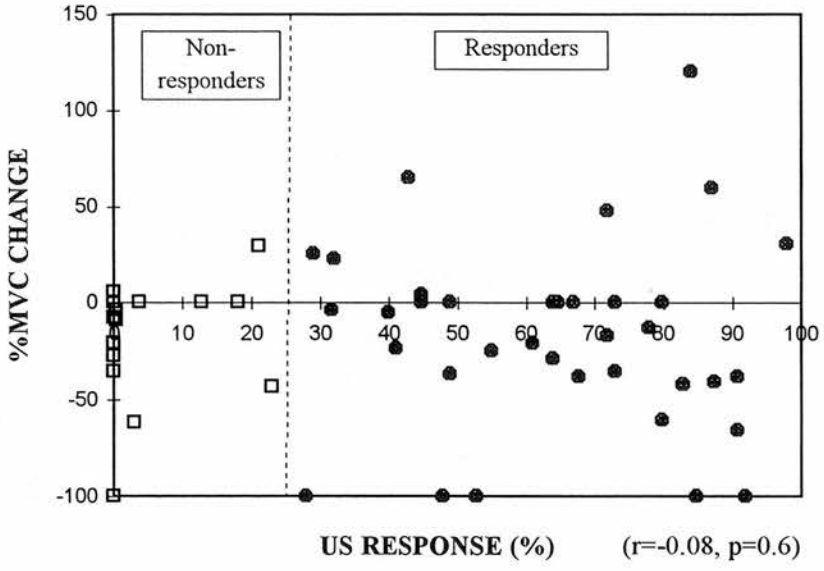
	Increased MVC	MVC static	Decreased MVC	TOTAL
Non-responders	3 (21%)	4 (29%)	7 (50%)	**14**
Responders	10 (23%)	7 (16%)	26 (61%)	**43**

Chi-squared test for trend: p=0.0062

Percentage reduction in tumour volume and change in microvessel count.

There was a significant reduction in mvc in responding tumours following staining with antibody to CD31. Figure 2.3.6 demonstrates no significant correlation between % change in mvc with treatment and degree of response as determined by ultrasound (r=-0.08, p=0.57). These results differ from those obtained following staining with antibody to Factor VIII, which demonstrated a correlation between the two of borderline statistical significance.

Figure 2.3.6: Relationship between degree of ultrasound response and % change in mvc with treatment.



Intensity of staining with antibody to CD31.

The staining intensity with antibody to CD31 varied between tumours and in 27 tumours (18% of total) sections persistently failed to stain despite repeating attempts on at least two occasions. Results described included sections which did not stain, in which microvessel counts were zero. In 7 patients, both pre- and post-treatment sections failed to stain, in 5 patients, pre-treatment sections only failed to stain and in 8, post-treatment sections only did not stain. Of the 8 post-treatment sections failing to stain, 7 occurred in responding tumours.

As the variation in staining intensity was thought to be due to methodological problems, analyses were performed excluding those patients in whom one or both of pre- and post-treatment tumours did not stain. Results obtained were not significantly different from those described. There was a significant difference in mvc following treatment in all patients ($p=0.016$) and in pre- and post-treatment mvc of responding tumours ($p=0.04$). Otherwise no differences were identified between responding and non-responding tumours as has been detailed in the previous section.

2.4 Comparison of counts performed following staining with Antibody to Factor VIII versus CD31 antibody.

Counts obtained following staining with antibody to Factor VIII and CD31 in the present study were compared. Both pre- and post-treatment mvc were included in the comparison. The relationship is illustrated on Figure 2.4.1 and demonstrates a statistically significant correlation between counts (r=0.21, p=0.01). Data are summarised on Table 2.4.1. When the sections which failed to stain with antibody to CD31 were excluded from the analysis, the correlation between counts was highly statistically significant (r=0.4, p<0.0001).

Figure 2.4.1: Comparison of counts performed following staining with antibody to Factor VIII and CD31.

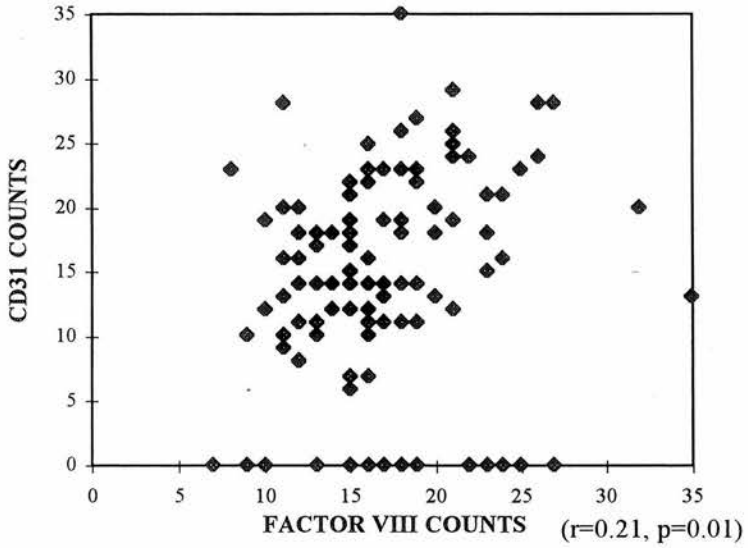


Table 2.4.1: Comparison of counts obtained following staining with Factor VIII and CD31 antibodies.

	Factor VIII antibody	CD31 antibody
Range	7-35	0-35
Mean	16.8	13.6
Median	16	14
Standard deviation	5.11	8.91
Standard error	0.48	0.84

Spearman correlation: r=0.21, p=0.01

Wilcoxon signed rank test: p=0.33

2.5 Microvessel counts and other tumour parameters.

Further analyses were performed to determine whether correlations existed between microvessel counts and lymph node involvement or level of oestrogen expression, factors which may affect tumour vascularity or response to treatment. Due to the failure of antibody to CD31 to stain vessels in a number of tumours, results in this section were based on staining with Factor VIII antibody

Lymph node involvement.

52 of the 57 patients underwent pathological assessment of lymph nodes. Five patients underwent surgery to the breast alone. The reasons for this were not clear on review of casenotes, but are likely to be due to the poor general medical condition of these patients. A total of 23 patients underwent axillary node sampling (6 non-responders and 17 responders) and 29 patients underwent axillary node clearance (7 non-responders and 22 responders). For the purpose of analysis, percentage of excised lymph nodes found to be positive for tumour was used to define lymph node involvement, as this took account of number of lymph nodes excised. Figure 2.5.1 illustrates the relationship between % lymph node involvement and response to tamoxifen. Median values of % lymph node involvement were 6% in non-responders and 3% in responders. The difference between the two groups was not significant (p=0.61).

Figure 2.5.1: % Lymph node involvement and response to tamoxifen.

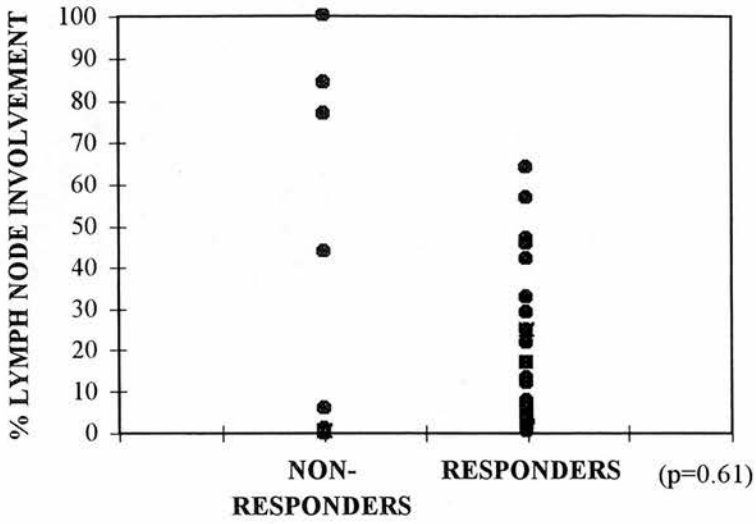


Table 2.5.1: Summary of data of % lymph node involvement and response:

	Non-responders	Responders
Range	0-100	0-100
Median	6	3
Mean	49.7	34.3
Standard deviation	171	43.6
Standard error	27.4	12.6

Mann-Whitney test: p=0.61

Lymph node involvement and microvessel counts.

Relationships between % lymph node involvement and tumour vascularity assessments are illustrated on Figures 2.5.2-4. There was no significant correlation between pre-treatment and % lymph node involvement as shown on Figure 2.5.2 (r=0.02, p=0.89). A significant correlation was identified between post-treatment mvc and % lymph node involvement (r=0.34, p=0.015), as shown on Figure 2.5.3. Both parameters reflect the tumour status following tamoxifen treatment. The correlation remained significant when expressed as % change in mvc following treatment and % lymph node involvement (r=-0.31, p=0.03) as shown on Figure 2.5.4, although this was less significant and so is likely to reflect the association between post-treatment mvc and lymph involvement.

Figure 2.5.2: Relationship between % lymph node involvement and pre-treatment mvc.

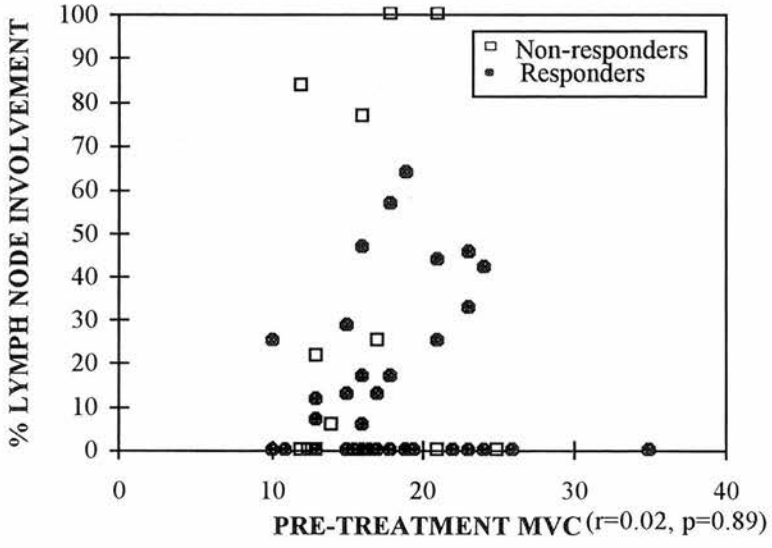


Figure 2.5.3: Relationship between % lymph node involvement and post-treatment mvc.

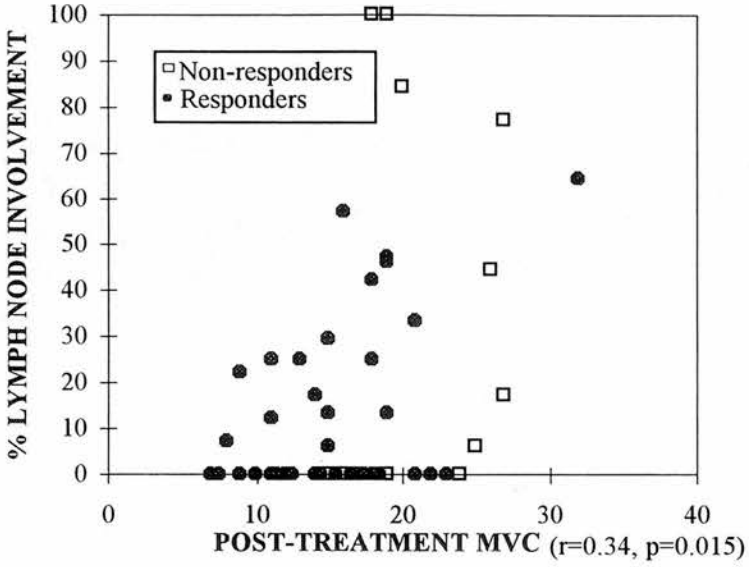
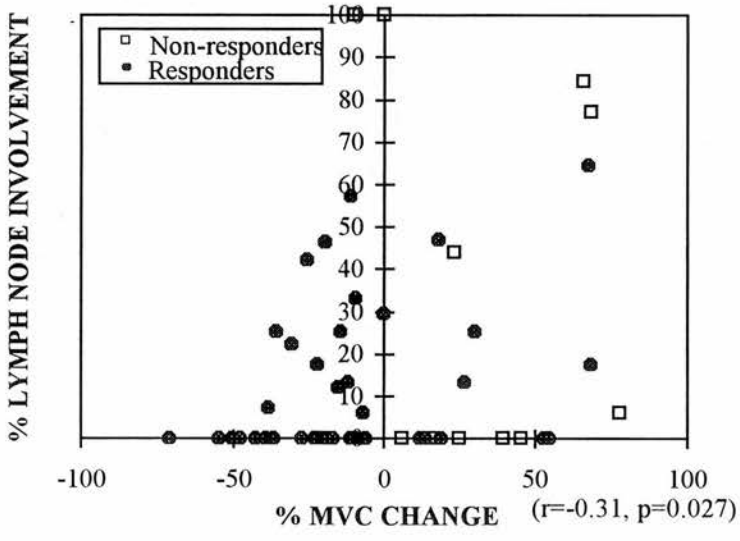


Figure 2.5.4: Relationship between % lymph node involvement and % change in mvc with treatment.



Levels of ER expression.

Pre-treatment oestrogen receptor levels (ER), assessed by biochemical methods, were available in all tumours and were initially compared with tumour response to treatment, as illustrated in Figure 2.5.5. Range of ER level in non-responding tumours was 25-673fmol/mg cytosolic protein (median: 111, mean: 180) and in responding tumours the range was 25-1496 fmol/mg cytosolic protein (median: 357, mean: 450). The difference between the two groups was statistically significant (p=0.0074).

Figure 2.5.5: Oestrogen receptor levels and response to tamoxifen.

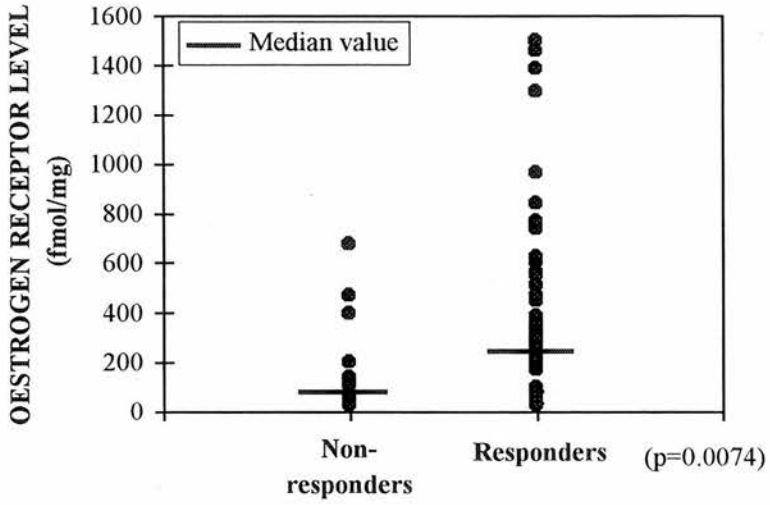


Table 2.5.2: Summary of data of ER level and response:

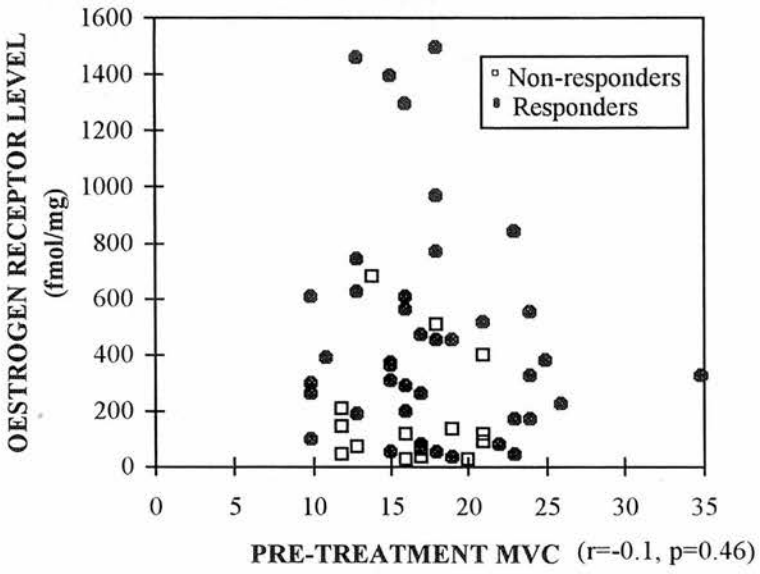
	Non-responders	Responders
Range	25-673	25-1496
Median	111	357
Mean	180	450
Standard deviation	195	389
Standard error	52	59

Mann-Whitney test: p=0.0074

ER levels and microvessel counts.

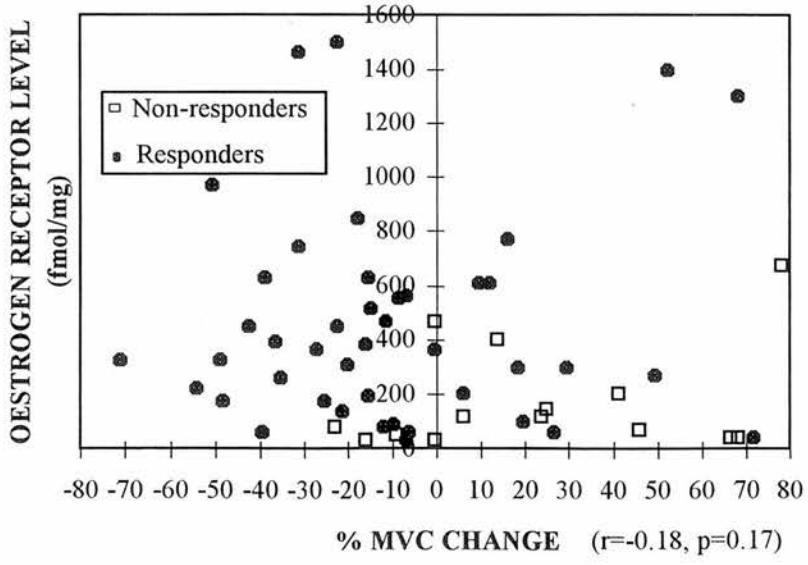
The relationship between ER level and pre-treatment mvc is illustrated on Figure 2.5.6, demonstrating no significant association between the two variables (r=-0.1, p=0.46). Both mvc and ER level were determined from the pre-treatment tumour sample, allowing comparison of the two variables at the same time point.

Figure 2.5.6: ER levels and pre-treatment mvc.



No significant association was demonstrated between ER level and % change in mvc, as illustrated in Figure 2.5.7.

Figure 2.5.7: ER level and % change in mvc with treatment.



2.6 Assessment of patients treated with tamoxifen for longer than six months.

15 patients underwent a longer period of treatment with neoadjuvant tamoxifen. The period of treatment ranged from 7 to 30 months (median: 10, mean: 11.8). Patients in this group had locally advanced disease with skin or muscle involvement or malignant ipsilateral axillary lymph nodes and response patterns varied when compared with the main group. In seven patients there was no evidence of response, three of these developed progressive disease; tumours in six patients responded initially, but then progressed, and in two patients response was maintained throughout treatment (7 and 9 months). For the purpose of the analysis, the response pattern of the tumour at the time of excision defined response: thus 13 are classified as non-responders, 2 as responders. Pre- and post-treatment mvc in non-responding and responding tumours are illustrated in Figure 2.6.1. Despite the complexity of the response, a similar pattern of change in mvc was seen in this subgroup as that observed in the main study.

Figure 2.6.1: Pre- and post-treatment mvc in patients treated for longer than six months.

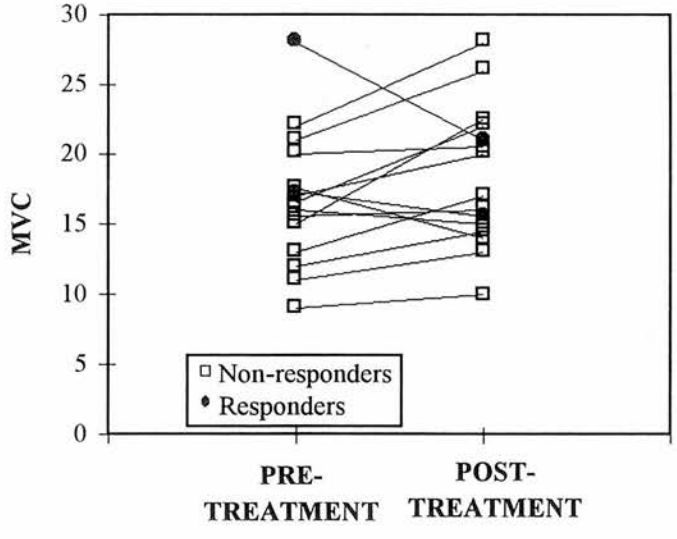


Table 2.6.1: Correlation of change in microvessel counts with treatment response:

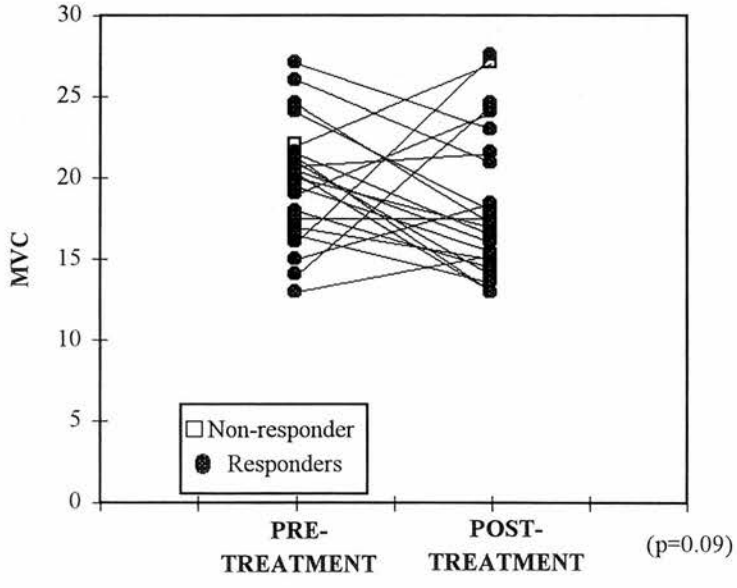
	Increased mvc	Mvc static	Decreased mvc	TOTAL
Non-responders	8	3	1	**12**
Responders	0	0	2	**2**

2.7 Changes in tumour vascularity in patients treated with neoadjuvant letrozole.

Letrozole is an aromatase inhibitor and offers an alternative strategy for primary endocrine therapy to tamoxifen. Twenty-two patients were treated with letrozole for three months prior to surgery and following a wedge biopsy for determination of ER status. Changes in tumour vascularity have been determined in these tumours to provide comparison with the effects of tamoxifen.

Twenty-one of the twenty-two patients responded to letrozole, with percentage reductions in tumour volume ranging from 33 to 96% (median: 70%, mean: 70.1%). Changes in mvc with response are illustrated in Figure 4.29, demonstrating a clear trend for reduced mvc in responding tumours and an increase in the non-responder. This just failed to reach statistical significance due to small numbers (p=0.09).

Figure 2.7.1: Changes in microvessel count following treatment with letrozole.



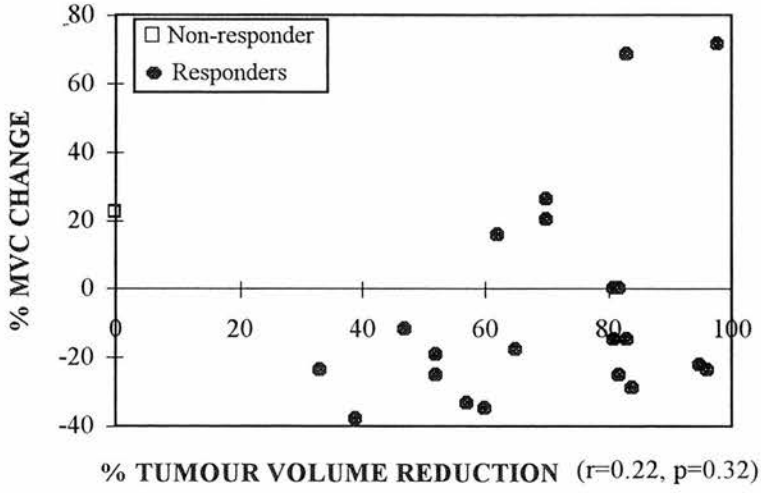
Correlation of changes in mvc with response to letrozole.

Numbers of patients with responding tumours in whom mvc decreased, increased or remained static are shown on Table 2.7.1. There was a reduction in mvc with treatment in the majority (67%) of patients with responding tumours, mvc increased in the non-responding tumour. The trend of change in mvc with response just failed to reach statistical significance (p=0.09), this is likely to be due to the small number of non-responding tumours in the group. The relationship between % reduction in tumour volume and % change in mvc with treatment is shown on Figure 4.30 and demonstrates no significant association between the two.

Table 2.7.1: Changes in mvc observed in patients treated with letrozole:

	Increased mvc	Mvc static	Decreased mvc
Responders	5 (24%)	2 (9%)	14 (67%)
Non-responder	1 (100%)	0	0

Figure 2.7.2: Relationship between % change in mvc and % reduction in tumour volume.



2.8 Discussion.

Fifty-seven post-menopausal patients with large, operable or locally advanced ER-positive primary breast cancer were treated with tamoxifen (20mg daily) for a period of between three and six months prior to surgery. Forty-three patients (75%) responded to tamoxifen with a greater than 25% reduction in tumour volume following treatment.

Assessments of tumour vascularity were made before and after treatment following staining with Factor VIII and CD31 antibodies on sequential tumour sections. Staining with antibody to CD31 varied in intensity between tumours with negative staining in 27 (18%); therefore, for completeness, discussion of the results will focus mainly on counts obtained following staining with antibody to Factor VIII. Possible explanations for the variation in intensity of CD31 staining will be addressed. Results of this study showed no difference in pre-treatment mvc between non-responding and responding tumours, indicating that the two groups were similar in terms of tumour vascularity at the outset. There was, however, a significant increase in post-treatment mvc in non-responding compared to responding tumours. When pre-and post-treatment mvcs were compared in responding tumours, there was a significant reduction in mvc following treatment. In non-responding tumours, treatment with tamoxifen was associated with increased mvc. There was a significant association between post-treatment mvc and % lymph node involvement and between % change in mvc and lymph node involvement. No significant associations were found between microvessel counts and level of ER expression.

Intensity of staining with antibody to CD31 varied between tumours, which had not been apparent following staining with antibody to Factor VIII. Twenty-seven tumours (24%) failed to stain despite repeated attempts on sequential sections: in 7 tumours both pre- and post-treatment sections failed to stain, 5 pre-treatment sections and 8 post-treatment sections failed to stain. In all of these tumours, vessels were identified on the H and E sections. It is of interest to note that, of the post-treatment sections failing to stain, 7 of the 8 were responding tumours. Whilst this suggests that tamoxifen treatment may result in a reduction in expression of the CD31 antigen (platelet endothelial cell adhesion molecule), failure of staining of pre-treatment sections suggests that a methodological problem is the more likely cause of the absence of staining. All sections were routinely processed in the pathology laboratory and sections that failed to stain did not correspond to those processed during a

specific period. Failure of a percentage of tumour sections to stain with CD31 antibody has been noted in another study which described an 87% success rate, with failure of staining attributed to antigen loss following tumour fixation (Martin *et al*, 1997). As the present study is a retrospective one, involving retrieval of tumour sections from pathology archives dating from 1990 to 1996, antigen loss due to prolonged storage is a possible explanation for negative staining. Factor VIII antibody stains more consistently than CD31, but also stains lymphatics (Horak *et al*, 1992; Martin *et al*, 1997). It has, however, been shown to provide useful prognostic information in a number of studies of breast cancer (Bosari *et al*, 1992; Toi *et al*, 1993; Weidner *et al*, 1992) and so has been used in addition to CD31 antibody in the present study.

Having defined a microvessel as any single stained endothelial cell or cell cluster, for those sections in which there was no staining with antibody to CD31 counts were taken as zero. Microvessel counts performed following staining with CD31 revealed a significant reduction in mvc following treatment with tamoxifen in responding but not non-responding tumours, in keeping with the previously described findings. However, no increase in mvc was observed in non-responding tumours following treatment.

Due to the disparity of staining patterns between sections stained with Factor VIII and CD31 antibody in this group of tumours, a direct comparison was made between counts obtained for all tumours (n=114). There was a highly significant correlation between counts, supporting findings described earlier in the thesis. Whilst counts obtained following Factor VIII staining (mean: 16.8, median: 16) tended to be higher than those following staining with CD31 (mean: 13.6, median: 14), no significant trend was observed (p=0.33). These differences are likely to reflect the presence of negatively staining sections in the CD31 group.

The response rate of 75% in the present study compared favourably with other studies (Allan *et al*, 1985; Anderson *et al*, 1991; Horobin *et al*, 1991), although an unconventional definition of response was used in the present study. The conventional method of assessment of tumour response adopts clinical measurements and is defined as a greater than 50% reduction in tumour volume maintained for at least one month. This definition was based on assessment of response in the context of metastatic disease and would not be practical in this study, in which patients are treated for three months alone. Ultrasonography is simple to perform, provides a permanent record of tumour size and may be carried out frequently, in

contrast to mammography. Ultrasound tumour volume assessments have been found to correlate more closely with pathological tumour size than clinical measurements (Forouhi *et al*, 1994), thus providing a practical and accurate method of monitoring response. Observer variation in volume assessed by ultrasound is less than 10% and thus a definition of response as >25% reduction in volume indicates a true reduction in volume that cannot be explained by observer variation alone (Forouhi *et al*, 1994). In addition, a cut-off of >50% reduction in tumour volume would have excluded patients from the responding group whose clinical management had been altered by treatment, rendering tumours amenable to conservation surgery instead of mastectomy. It was for these reasons that a cut-off point of >25% reduction in tumour volume was adopted in the present study to define a responding tumour, although the continuous variable of percentage tumour volume reduction was also used in the analysis.

Changes in tumour vascularity in association with response to primary tamoxifen therapy have not been previously investigated in the clinical setting. However, the finding of a fall in mvc observed in responding but not non-responding tumours is in keeping with experimental evidence that oestrogen deprivation causes reduced vascularity (Morales *et al*, 1995; Sypridopoulos *et al*, 1997), and that tamoxifen may exert an anti-angiogenic effect (Gagliardi *et al*, 1995). In the present study, whilst a reduction in mvc was observed in the majority (63%) of responding tumours, there was a subgroup (22%) in whom mvc increased. This disparity may reflect the problems inherent in observing a dynamic process at a single time point. Angiogenesis is stimulated by tissue hypoxia (Hlatky *et al*, 1994), which may occur for a period of time within a responding tumour. This provides a possible explanation for the increased mvc detected in 22% of responding tumours following treatment. In addition, previous surgical manipulation of the tumour is likely to stimulate angiogenesis as part of the process of wound healing; the duration of such an effect is unknown.

The pattern of change in tumour vascularity observed in non-responding tumours is also of interest; mvc increased in the majority (57%) of these tumours, with counts remaining static in only 3 tumours (21.5%) and decreasing in a further 3 tumours. It is recognised that increasing tumour vascularity is a component of untreated tumour growth; a switch to the angiogenic phenotype is accompanied by rapid tumour growth and progression (Hanahan & Folkman, 1996). Oestrogen exerts a pro-angiogenic effect and it has been suggested that, under certain conditions such as low concentration, tamoxifen may act as an oestrogen agonist (Osborne *et al*, 1992). Thus it is possible that tamoxifen may actually stimulate

angiogenesis in some non-responding tumours. Such increases in vascularity may also reflect the development of a resistant phenotype. However, the increased mvc observed in the majority of non-responding tumours may simply reflect methodological issues, such as the effect of previous surgical manipulation and tumour heterogeneity. These issues have been addressed elsewhere in the thesis.

Information about pathological lymph node status was available in 90% of patients in the study. No correlation was found between % lymph node involvement and response, or between % lymph node involvement and pre-treatment mvc. This is unexpected as microvessel counts have been found to provide independent information regarding lymph node metastasis in primary breast cancers treated surgically (Horak *et al*, 1992). The significant correlations between % lymph node involvement and both post-treatment mvc and % change in mvc are interesting, particularly in the light of the lack of correlation with pre-treatment assessments of angiogenesis. Correlation of lymph node involvement and post-treatment mvc is slightly more significant ($p=0.015$) than the correlation with % mvc change ($p=0.027$), suggesting that it is post-treatment mvc which is more influential. Post-treatment mvc and pathological lymph node status were both determined at surgery and so reflect the nature of the tumour at the same time point. Clinical assessment of lymph node status is unreliable and therefore accurate knowledge of lymph node status prior to neoadjuvant treatment is not available. Thus, the effect of tamoxifen on involved lymph nodes is not known, but it is possible that, in responding tumours, tamoxifen may render some initially positive lymph nodes free from tumour.

There was a significant correlation between level of oestrogen receptor (ER) expression and response (Figure 2.5.5) which supports findings of other studies (Gaskell *et al*, 1992; Osborne *et al*, 1980). The lack of association between ER levels and pre-treatment mvc or % mvc change is worthy of note. The possible mechanisms of action of tamoxifen on tumour vascularity have not been addressed in this study as observations of change in vascularity have been made at an individual time point at the end of the treatment course. It has been suggested that tamoxifen may exert an anti-angiogenic effect independent of the oestrogen receptor (Gagliardi & Collins, 1993) and the lack of correlation between mvc and ER supports this finding. However, it must be noted that all ER-negative and ER-poor tumours were excluded from the present study.

In order to determine whether such changes in vascularity were specific to tamoxifen, a control group of 22 patients with tumours treated with the aromatase inhibitor, letrozole, was also studied. Although the patient number is small, precluding extensive analysis, a similar pattern of change in mvc in responders and non-responders was seen. There is no experimental evidence to suggest that letrozole exerts a direct anti-angiogenic effect, suggesting that observed changes in tumour vascularity may be a reflection of either oestrogen deprivation or a component of tumour response to any form of systemic therapy.

One of the shortcomings of the study is that all observations were made at the end of the period of neoadjuvant therapy, when clinical response to treatment was already apparent. Whether the observed changes in vascularity occur as a result of tumour response, or precede it and contribute to the process of response, cannot be ascertained.

These studies have identified a significant correlation between tumour response to primary endocrine therapy and changes in tumour vascularity. The timing of such changes cannot be assessed using the current study design. If such changes occurred early in treatment, they may provide a useful early predictor of response, which would be of great value in the clinical setting. This issue is addressed in the later studies within this thesis.

3. Chronology of changes in tumour vascularity and response to tamoxifen: a study of breast cancer xenografts.

Results described in the previous chapter demonstrated a change in tumour vascularity in association with response to primary tamoxifen treatment. A reduction in microvessel density was observed in responding tumours and an increase non-responding tumours. Such changes were observed after between three and six months of primary endocrine treatment, when clinical response was already apparent. It would be of benefit in the clinical setting if such changes predated clinical evidence of response when they would provide a predictive marker of sensitivity to hormone therapy.

The aim of the present series of experiments was to determine the chronology of changes in tumour vascularity relative to tumour response to tamoxifen. In order to achieve this aim xenograft model systems have been studied, allowing accurate monitoring of changes in tumour volume and assessments of tumour vascularity at several time points.

Experiments were performed involving the oestrogen receptor-positive breast cancer cell line, ZR-75, and the oestrogen receptor-negative breast cancer cell line, MDA-MB-231. Tumours were implanted into the flanks of nude mice bearing slow release oestrogen pellets and were randomly allocated into treatment and control groups. Slow-release tamoxifen pellets (0.25mg over 60 days) were inserted alongside tumours of animals in the treatment groups.

Tumours were excised from animals at the time points described in Table 3.1, allowing assessment of tumour vascularity on 2, 4, 7 and 14 days following commencement of tamoxifen.

Table 3.1: Number of tumours excised at each time point:

DAY	0	2	4	7	14
ZR-75: control	4	0	0	4	0
ZR-75: Treated	0	4	4	4	0
MDA-MB-231: control	4	0	0	4	5
MDA-MB-231: treated	0	4	4	4	4

Response data on tumours are expressed as the mean relative tumour volume for each time point before and during treatment. Tumour volumes were calculated according to the equation:

$\pi/6.\ d\ xD^2$ where D is the larger of the two diameters.

Tumour volume for each time point was calculated relative to the volume at day 0, and expressed as a percentage of this. Mean relative tumour volumes were calculated for each group and these were used in the analysis.

Microvessel counts were performed on tumour sections excised after 2, 4,7 and 14 days of treatment following staining with the endothelial cell marker, MEC 13.3, and compared with counts in tumours from the control groups.

3.1. Tumour growth: ZR-75 xenografts.

The growth curve of ZR-75 tumours in the treatment and control groups is illustrated on Figure 3.1.1. Tumour volume is expressed as a percentage of the volume at day 0 (100%) and demonstrated no difference between the two groups prior to commencement of tamoxifen. A progressive increase in size was observed in both the control and treated tumours until day 4, following which evidence of tumour regression became apparent in the treated tumours. Differences in mean relative tumour volume between treated and control tumours failed to reach statistical significance. Mean relative tumour volumes at each time point are outlined in Table 3.1.1, with details of individual tumour volume described in the Appendix to this chapter.

Figure 3.1.1: Anti-tumour effect of tamoxifen against ZR-75 breast carcinoma xenografts.

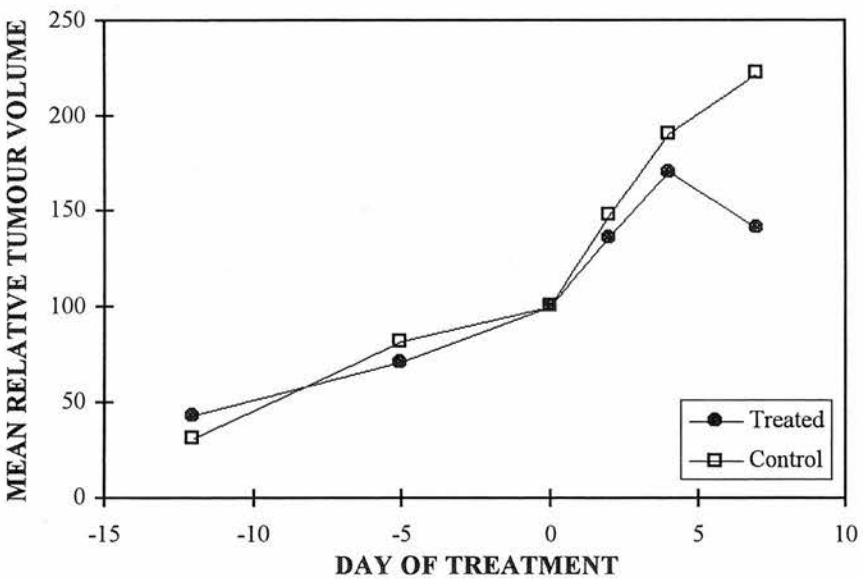


Table 3.1.1: Mean relative tumour volume of treated and control ZR-75 tumours, expressed as a percentage of volume at Day 0: (+/-standard error of mean)

ZR-75	DAY -12	DAY -5	DAY 0	DAY 2	DAY 4	DAY 7
TREATED	43	71	100	136 (+/-8.2)	170 (+/-11.8)	140 (+/-21.3)
CONTROL	31	82	100	147 (+/-21.4)	191 (+/-13.3)	222 +/-14.0)

Mann-Whitney tests:

Tumour volume at Day 2: treated v. control groups: p=0.73

Tumour volume at Day 4: treated v. control groups: p=0.28

Tumour volume at Day 7: treated v. control group: p=0.11

3.2. Tumour growth: MDA-MB-231 xenografts.

The growth curve of treated compared with control MDA-MB-231 tumours is illustrated on Figure 3.2.1 and shows that the growth curves follow a very similar pattern in the two groups throughout treatment. There was no evidence of tumour regression in either treated or control group in contrast to the ZR-75 tumours. Data are summarised on Table 3.2.1.

Figure 3.2.1: Lack of anti-tumour effect of tamoxifen on MDA MB-231 breast cancer xenografts.

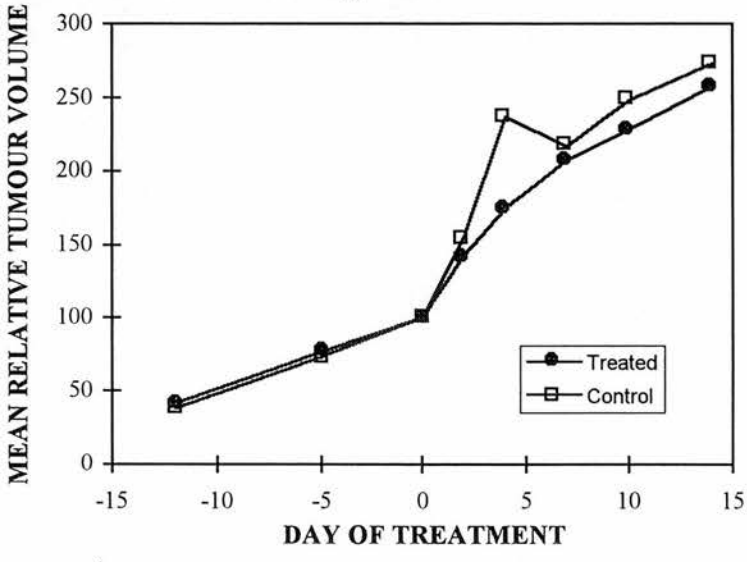


Table 3.2.1: Mean relative tumour volume at each time point of treated and control MDA-MB-231 tumours: (+/-standard error of mean)

MDA-MB-231	DAY -12	DAY -5	DAY 0	DAY 2	DAY 4	DAY 7	DAY 10	DAY 14
TREATED	41	77	100	142 (+/-7.3)	175 (+/-6.7)	208 (+/-23)	228 (+/-32.5)	258 (+/-34.4)
CONTROL	38	73	100	155 (+/-12.0)	237 (+/-47.3)	217 (+/-26.5)	248 (+/-28.4)	273 (+/-32.1)

Mann-Whitney tests: Tumour volume at Day 2: treated v. control groups: p=0.34

Tumour volume at Day 4: treated v. control groups: p=0.13

Tumour volume at Day 7: treated v. control group: p=0.72

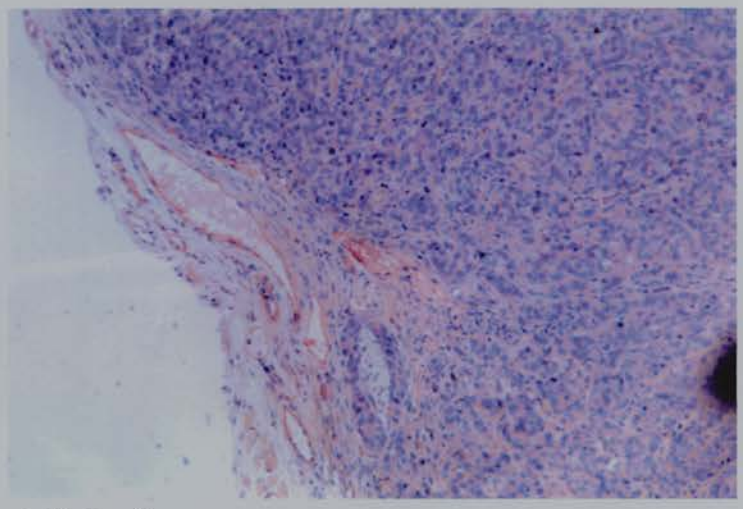
Tumour volume at Day 10: treated v. control group: p=0.91

Tumour volume at Day 14: treated v. control group: p=0.91

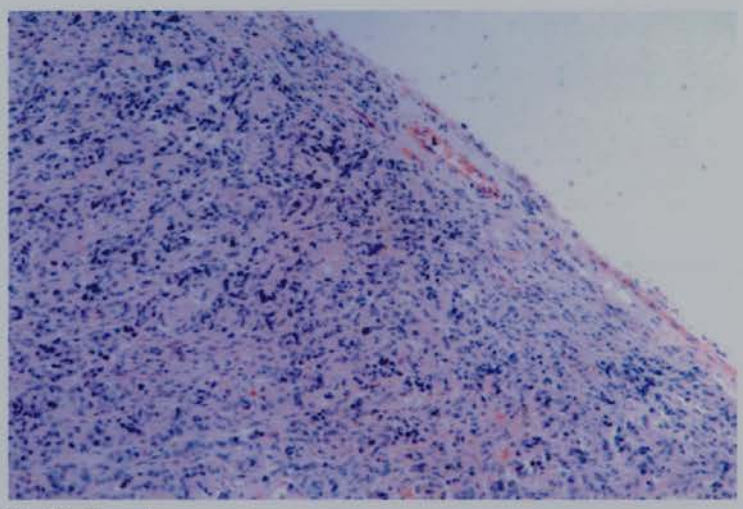
3.3. Microvessel counts in treated and control ZR-75 xenografts.

Microvessel counts were performed on all tumours in treated and control groups following staining with MEC 13.3, the murine equivalent of antibody to CD31. The majority of vessels were situated at the periphery of the tumours as illustrated on Figure 3.3.1. Mean microvessel counts (mvc) in treated versus control tumours are shown on Figure 3.3.2, demonstrating a progressive reduction in mvc during tamoxifen treatment. This was already apparent following two days of treatment ($p=0.03$). It should be noted, however, that the difference in mvc between treated and control tumours at day 7 was not significant ($p=0.32$). Data are outlined in Table 3.3.1.

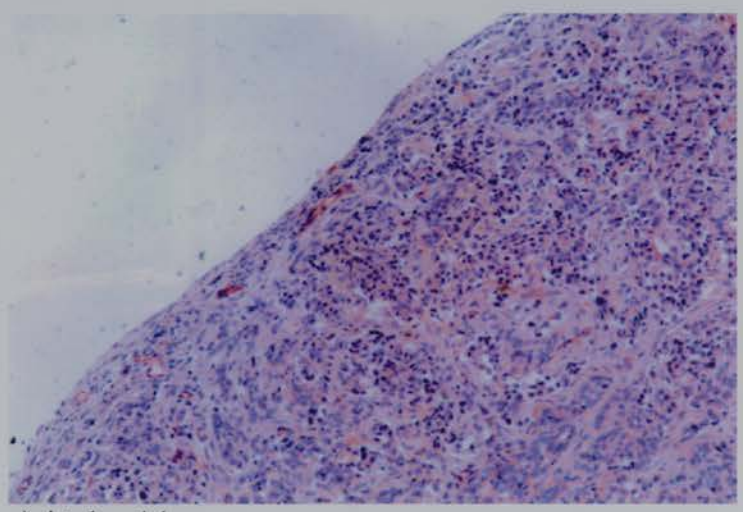
Fig 3.3.1. Treated ZR-75 tumour following staining with MEC13-3 antibody



a) At day 0



b) At day 4



c) At day 14

Figure 3.3.2: Changes in microvessel count in ZR-75 breast cancer xenografts during treatment with tamoxifen.
(p value: Day 0 control v. Day 2 treated tumours).

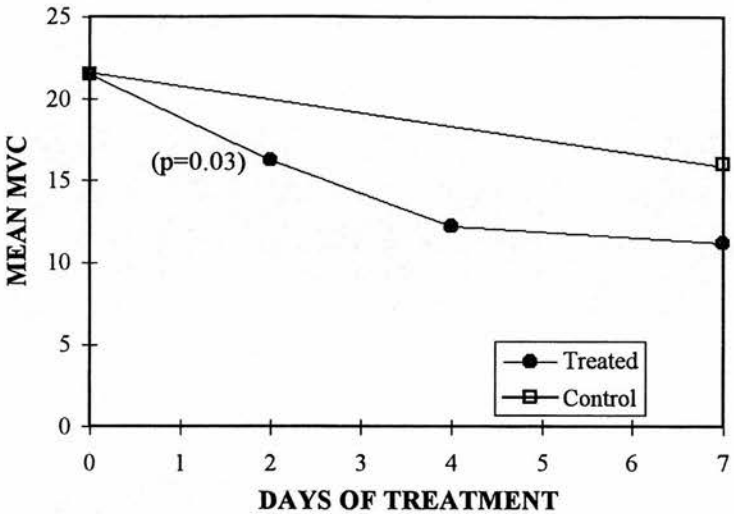


Table 3.3.1: Microvessel counts in treated and control ZR-75 tumours at different time points:

Day	Microvessel counts	Standard error	Standard deviation
DAY 0 CONTROL			
634 ZR	24		
651 ZR	27		
108 ZR	20		
645 ZR	20		
MEAN	**21.5**	**1.7**	**3.4**
DAY 2 TAMOXIFEN			
105 ZR	12		
624 ZR	17		
633 ZR	18		
103 ZR	18		
MEAN	**16.25**	**1.4**	**2.9**
DAY 4 TAMOXIFEN			
104 ZR	13		
641 ZR	10		
627 ZR	11		
106 ZR	15		
MEAN	**12.25**	**1.1**	**2.2**
DAY 7 TAMOXIFEN			
622 ZR	16		
638 ZR	5		
621 ZR	10		
107 ZR	14		
MEAN	**11.25**	**2.4**	**4.9**
DAY 7 CONTROL			
629 ZR	23		
623 ZR	10		
650 ZR	15		
MEAN	**16**	**3.8**	**6.6**

Mann-Whitney tests:

Microvessel counts:

Day 2 treated v. Day 0 control: p=0.027

Day 4 treated v. Day 0 control: p=0.0021

Day 7 treated v. Day 0 control: p=0.00082

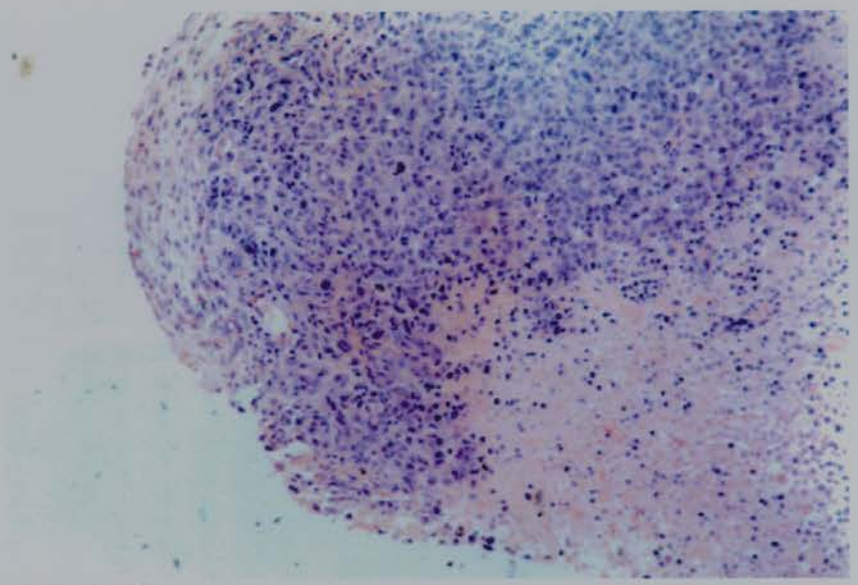
Day 0 control v. Day 7 control: p=0.13

Day 7 treated v. Day 7 control: p=0.32

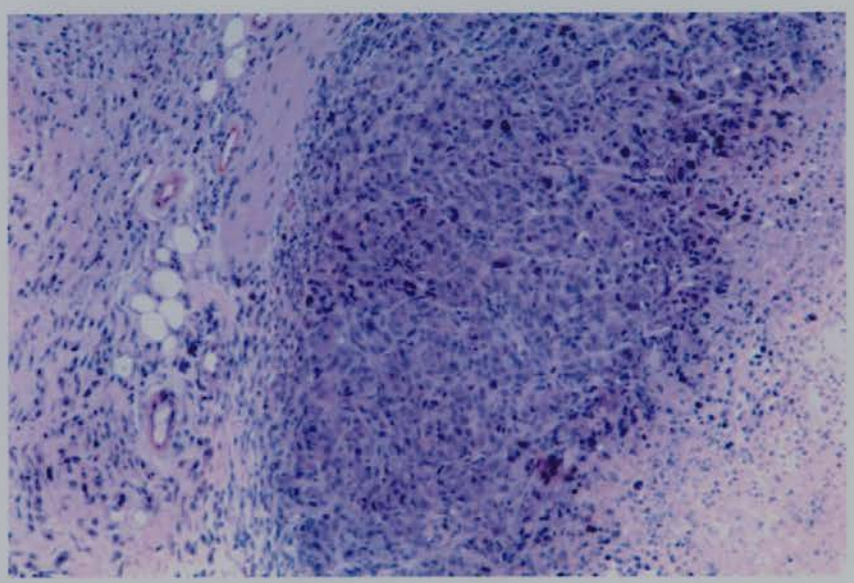
3.4. Microvessel counts in treated and control MDA-MB-231 tumours.

Staining of MDA-MB-231 tumour sections with MEC13.3 antibody is illustrated in Figure 3.4.1. Microvessel counts in treated and control tumours are compared in Figure 3.4.2, demonstrating no significant difference between the two groups or between Day 0 control and treated tumours. Microvessel counts appeared to decrease in control tumours compared with treated tumours, but the difference was not significant ($p=0.73$). Data are outlined on Table 3.4.1.

Fig 3.4.1. Treated MDA-MB-231 tumours following staining with MEC13-3 antibody



a) At day 0



b) At day 14

Figure 3.4.2: Comparison of microvessel counts in treated and control groups of MDA-MB-231 tumours.
(p value: Day 7 treated v. Day 7 control)

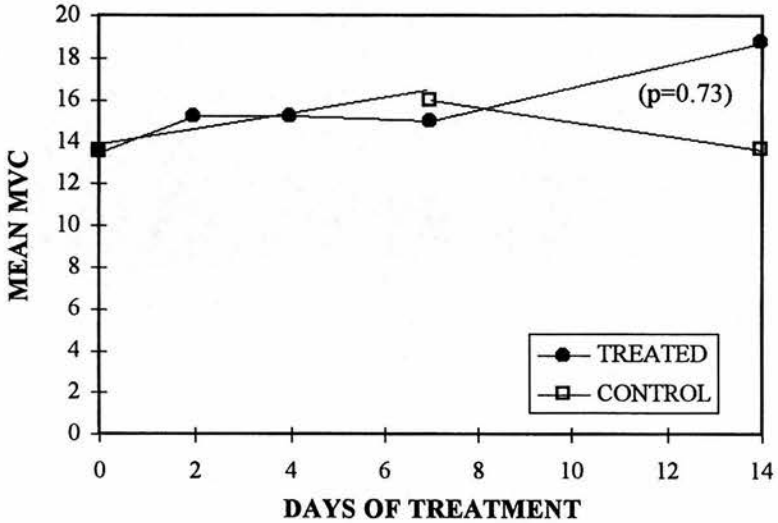


Table 3.4.1: Comparison of microvessel counts in treated and control MDA-MB-231 tumours:

Day	Microvessel counts	Standard error	Standard deviation
DAY 0 CONTROL			
634 MDA	9		
645 MDA	18		
651 MDA	16		
108 MDA	11		
MEAN	**13.5**	**2.1**	**4.2**
DAY 2 TAMOXIFEN			
105 MDA	18		
624 MDA	18		
633 MDA	11		
635 MDA	14		
MEAN	**15.25**	**1.7**	**3.4**
DAY 4 TAMOXIFEN			
104 MDA	14		
631 MDA	14		
627 MDA	13		
106 MDA	20		
MEAN	**15.25**	**1.6**	**3.2**
DAY 7 TAMOXIFEN			
622 MDA	14		
638 MDA	18		
621 MDA	16		
107 MDA	12		
MEAN	**15**	**1.3**	**2.6**
DAY 14 TAMOXIFEN			
628 MDA	32		
615 MDA	16		
620 MDA	15		
632 MDA	12		
MEAN	**18.75**	**4.5**	**9**

Day	Microvessel counts	Standard error	Standard deviation
DAY 7 CONTROL			
629 MDA	12		
617 MDA	21		
643 MDA	15		
MEAN	**16**	**2.7**	**4.6**
DAY 14 CONTROL			
616 MDA	16		
646 MDA	11		
102 MDA	17		
636 MDA	19		
101 MDA	5		
MEAN	**13.6**	**2.5**	**5.6**

Mann-Whitney tests:

Microvessel counts: Day 2 treated v. Day 0 control: p=0.49

Day 4 treated v. Day 0 control: p=0.69

Day 7 treated v. Day 0 control: p=0.69

Day 14 treated v. Day 0 control: p=0.49

Day 0 control v. Day 7 control: p=0.63

Day 0 control v. Day 14 control: p=0.91

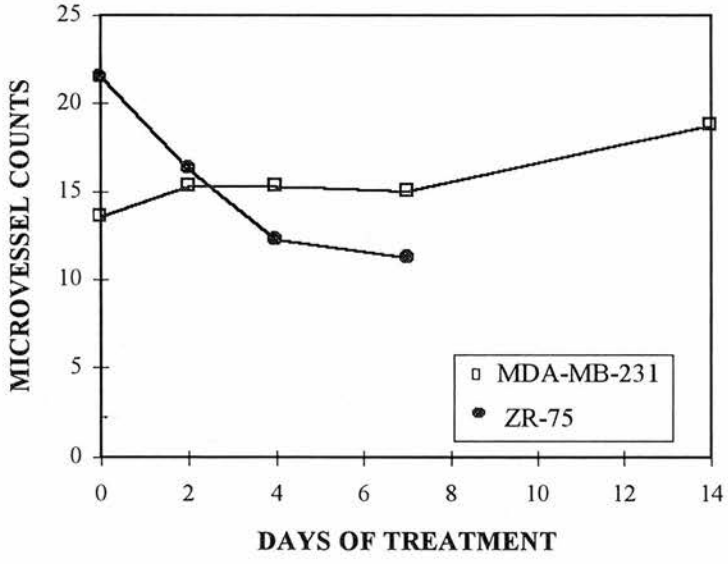
Day 7 treated v. Day 7 control: p=0.4

Day 14 treated v. Day 14 control: p=0.73

3.5. Comparison of microvessel counts in ZR-75-1 and MDA-MB-231 tumours during treatment with tamoxifen.

Figure 3.5.1 illustrates microvessel counts in ZR-75-1 compared with MDA-MB-231 tumours during treatment with tamoxifen, and demonstrates a reduction in mvc at Day 2 in ZR-75 tumours but not in MDA-MB-231 tumours. Microvessel counts in pre-treatment ZR-75 tumours were higher than MDA-MB-231 (p=0.03), reflecting differing characteristics of the tumour types.

Figure 3.5.1: Comparison of microvessel counts in treated ZR 75 and MDA-MB-231 tumours.



Mann-Whitney test:

Microvessel counts: ZR-75-1 v. MDA-MB-231 tumours: Day 0: p=0.029

Day 7: p=0.34

3.6. Chronology of changes in microvessel count relative to tumour volume.

Figure 3.6.1 compares timing of changes in mvc and relative tumour volume during treatment with tamoxifen of ZR-75 xenografts. Microvessel counts are expressed as a percentage of mvc at Day 0. The figure demonstrates that the reduction in mvc occurred as early as two days into treatment with tamoxifen, whereas tumour regression only became apparent after four days. Figure 3.6.2 demonstrates a similar comparison of changes in MDA-MB-231 tumours during tamoxifen treatment and illustrates the lack of effect of tamoxifen on both tumour growth and microvessel counts.

Figure 3.6.1: Graph to demonstrate timing of changes in relative tumour volume and microvessel counts in ZR-75 xenografts.

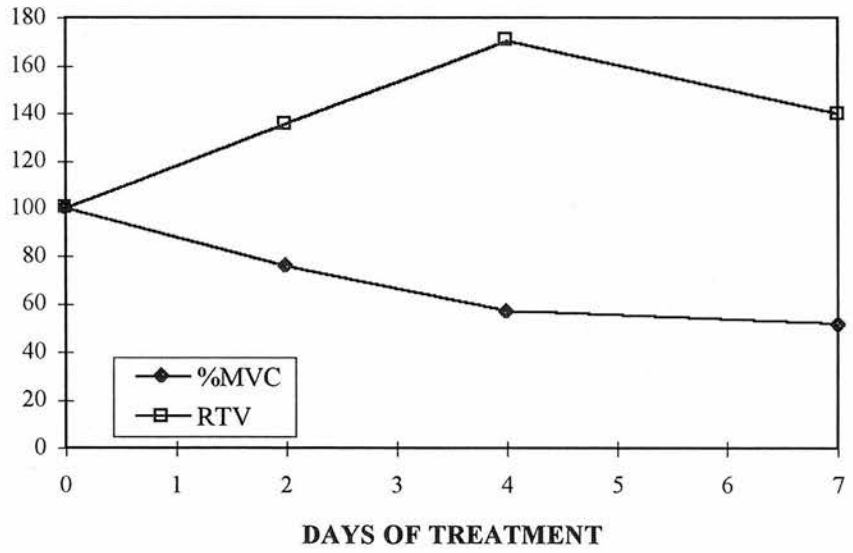
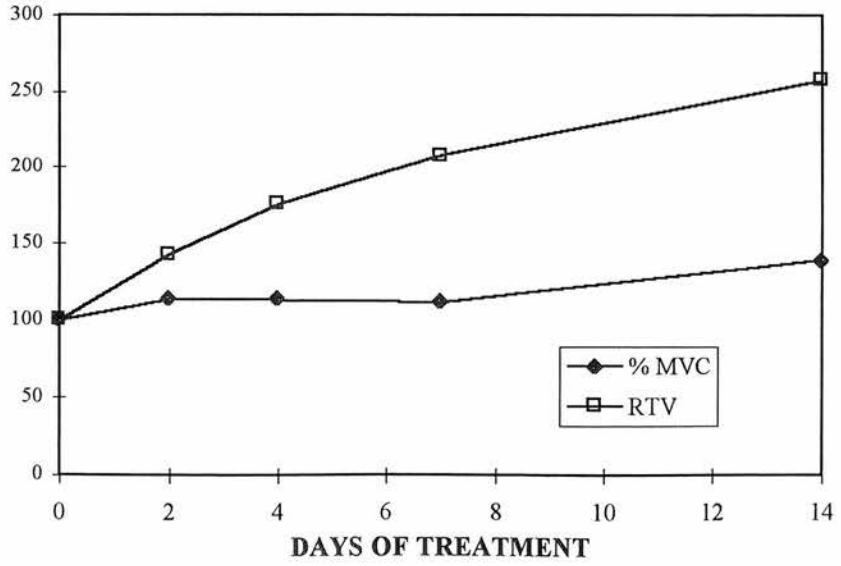


Figure 3.6.2: Graph to demonstrate timing of changes in relative tumour volume and microvessel counts in MDA-MB-231 tumours.



3.7. Discussion.

A clear association between change in tumour vascularity and response to neoadjuvant endocrine therapy in primary breast cancers was demonstrated in patients treated with tamoxifen, with a reduced vascularity in responding tumours and an increased vascularity in non-responding tumours. The aims of the present study were to confirm that changes in tumour vascularity occurred in association with response to primary tamoxifen treatment using a xenograft model system and to determine the chronology of such changes relative to response.

The ER-positive ZR-75 breast cancer xenografts demonstrated a clear regression of tumour volume between four and seven days following commencement of treatment with tamoxifen, compared with control tumours, although the differences were not significant, probably due to small numbers. Such an effect was not observed in the ER-negative MDA-MB-231 xenografts, in which similar growth rates were observed in treated and control tumours. Microvessel counts (mvc) were performed following staining with MEC 13.3, the murine equivalent of antibody to CD31, at several time points during tamoxifen treatment. There was a significant reduction in mvc following 2 days of treatment of ZR-75 tumours compared with counts of Day 0 control tumours ($p=0.03$). Microvessel counts in this group continued to decrease throughout the duration of the experiment (7 days). Although there was a fall in mvc in control tumours observed at Day 7 compared with Day 0, this difference was not significant and only three tumours were available for assessment as Day 7 controls. There was no difference in mvc between treated and control groups of MDA-MB-231 tumours, with a tendency for mvc to increase in treated tumours. This reflects similar changes to those described in primary breast cancers, with an increased mvc in non-responding tumours. The lack of response in the ER-negative tumours is interesting as it has been suggested that tamoxifen may exert a direct anti-angiogenic effect, independent of its action as an ER-antagonist (Gagliardi & Collins, 1993). These findings showed that tamoxifen failed to exert an anti-angiogenic effect on the ER-negative MDA-MB-231 tumours.

Comparison of mvc in ZR-75 and MDA-MB-231 tumours demostrated a higher pre-treatment mvc in the ZR-75 tumours ($p=0.029$). This is likely to reflect a difference in angiogenic profiles between the two tumours. In the clinical setting, there is marked variation in mvc between different tumours, and mvc has been shown to provide independent

significant prognostic information in breast cancer (Fox *et al*, 1994). ER-positive ZR-75 tumours are likely to be less aggressive than ER-negative tumours and so would be expected to have a lower pre-treatment mvc. However, the opposite was demonstrated in this study.

The relative timing of changes in tumour growth and vascularity in the ZR-75 tumours are of interest. Reduction in mvc had reached statistical significance by day 2, whereas tumour regression only became apparent in the period between 4 and 7 days of treatment. These changes suggest that reduction in vascularity does indeed precede tumour regression and may be an early marker of sensitivity to primary endocrine therapy.

Results of this study were similar to those found in another study performed in the Edinburgh Breast Unit (Cameron *et al*, 1996). In this study, the same antibody was used on frozen sections, and required immunofluorescence for antibody visualization. This prohibited the use of a standard counting method such as the Chalkley count described in the present study. In addition, in the previous study, the majority of vessels counted lay within the central area of tumour, whereas in the present study, vascular 'hot spots' were found mainly at the periphery. Nevertheless, a reduction in mvc was observed in this previous study following 2 days of treatment of ZR-75 tumours with tamoxifen, but not in the MDA-MB-231 tumours (Cameron *et al*, 1996). As in the present study, such changes preceded tumour regression.

Other studies investigating the effect of tamoxifen on vascularity in breast cancer xenografts have demonstrated similar results, but tumours were not assessed at early time points. Changes in mvc have been demonstrated concomitant with tumour regression (Furman-Haran *et al*, 1994; Lindner & Borden, 1997). In one study of MCF-7 breast cancer xenografts, tumour growth was stimulated by oestrogen alone or inhibited with tamoxifen following removal of the oestrogen pellet (Furman-Haran *et al*, 1994). A third group was treated with placebo following removal of the oestrogen pellet, but assessments of endothelial cell density were not performed in this group. Tumour growth arrest was observed after one week in the tamoxifen-treated group and tumour regression was seen following two weeks of treatment. Microvessel counts were performed following two weeks of treatment and were reduced in the tamoxifen treated group. However, as the oestrogen pellet was removed at the time of insertion of tamoxifen, such changes may be due to a combination of removal of oestrogen stimulation and tamoxifen inhibition (Furman-Haran *et al*, 1994). In this study, patterns of endothelial cell growth similar to those observed in the

present study were described, with larger vessels at the periphery (Furman-Haran *et al*, 1994).

In a similar study of MCF-7 xenografts, treatment with tamoxifen for ten weeks resulted in a reduction in the number of vessels by 68% and inhibition of tumour growth by 67% (Lindner & Borden, 1997). Changes in vessel count in treated tumours were observed following 48 hours of treatment and reached statistical significance by 4 days. Inhibition of angiogenesis was observed prior to tumour regression, in keeping with the findings of the present study. Changes in tumour growth and vascularity in MCF-7 tumours were compared with the oestrogen-independent NIH-OVCAR-3 ovarian cancer cell line. In the latter tumours, angiogenesis was also effectively inhibited by tamoxifen, suggesting that inhibition of angiogenesis may have occurred independently of the oestrogen receptor (Lindner & Borden, 1997). Reduced angiogenesis and tumour volume were observed much later in the NIH-OVCAR-3 ovarian tumours than the MCF-7 tumours. This contrasts with findings of the present study, in which there was no apparent reduction in tumour volume or vascularity in ER-negative tumours over a period of two weeks' treatment.

In summary, this study of breast cancer xenografts treated with tamoxifen demonstrated tumour regression in ER-positive ZR-75 tumours, but not in ER-negative MDA-MB-231 tumours. Microvessel counts were reduced in ZR-75 tumours following 2 days of tamoxifen treatment and preceded evidence of growth arrest or tumour regression. There was no reduction in the mvc of MDA-MB-231 tumours. These results suggest that reduction in tumour vascularity is an early event in the process of response to tamoxifen and that it may provide an early marker of sensitivity to primary endocrine treatment.

4. Sequential changes in tumour vascularity during treatment with tamoxifen: a prospective study.

Results in this thesis have demonstrated changes in tumour vascularity during response to primary endocrine treatment. Xenograft studies have suggested that these may occur early during treatment and thus may provide a useful marker of response prior to clinical evidence. In an earlier chapter, problems with reproducibility of tumour vascularity assessments in small tumour biopsies were demonstrated, which may not necessarily be problematic if the magnitude of effect of primary treatment on vascularity is sufficiently great. The aim now is to determine whether changes in tumour vascularity detected early in treatment can provide a useful marker of response before this becomes clinically apparent. Results of a prospective study are described, in which patients underwent tumour biopsies early during treatment.

Fifty-five patients with large, operable or locally advanced breast cancer were recruited into this prospective study. Patients underwent an initial core biopsy to obtain a diagnosis and determination of oestrogen receptor (ER) level. They were then commenced on tamoxifen (20mg daily) for a period of three months. Following two weeks of tamoxifen treatment core biopsies were repeated. Patients were monitored with monthly clinical and ultrasound assessments of tumour volume. In addition nine of the fifty-five patients were assessed by serial colour Doppler ultrasound, which provides a non-invasive method of assessing tumour vascularity. Forty-two patients completed the study: reasons for the failure of completion in thirteen patients are described later.

Response data of these patients were based on changes in clinical and ultrasound assessments of tumour volume during treatment and tumour vascularity was assessed by microvessel counts performed following staining with antibody to CD31 and Factor VIII. Relationships between response and tumour vascularity data were determined. In addition, lymph node involvement and level of oestrogen expression was correlated with response and with microvessel counts. Finally, data available in nine patients who underwent sequential colour Doppler ultrasound scans throughout treatment were described.

4.1. Clinical assessment of tumour response to tamoxifen.

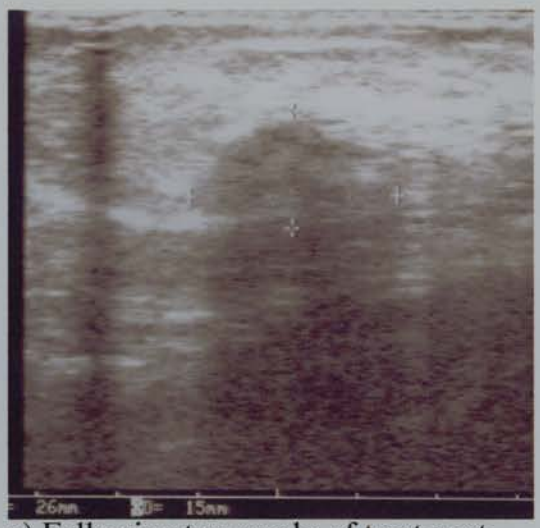
Patients were assessed following treatment with tamoxifen for two, six and twelve weeks. Tumour volume was assessed by clinical caliper and ultrasound measurements of anteroposterior and transverse diameters and depth. In addition, mammography was performed before and after three months of treatment. Ultrasound assessments of tumour volume have been found to provide most accurate information as described previously. Therefore, in those patients with three available ultrasound measurements of tumour volume, percentage reduction in tumour volume was calculated from these (n=36). In six patients such information was not available: one patient had an ulcerating tumour, which precluded use of ultrasound. In three of these patients, clinical assessments only were available for each time point, because of technical problems with the ultrasound scanner. In another two patients, casenotes were not available, so tumour response had to be assessed based on mammographic evidence.

Examples of serial ultrasound assessments of a responding tumour are illustrated on Figure 4.1.1 and mammographic evidence of response is shown on Figure 4.1.2. Serial changes in tumour volume in all patients are shown on Table 4.1.1, demonstrating a wide range of response to tamoxifen over three months. The median reduction in tumour volume at six weeks was 11% (mean: 25%) and at 12 weeks was 46% (mean 40%) for all patients.

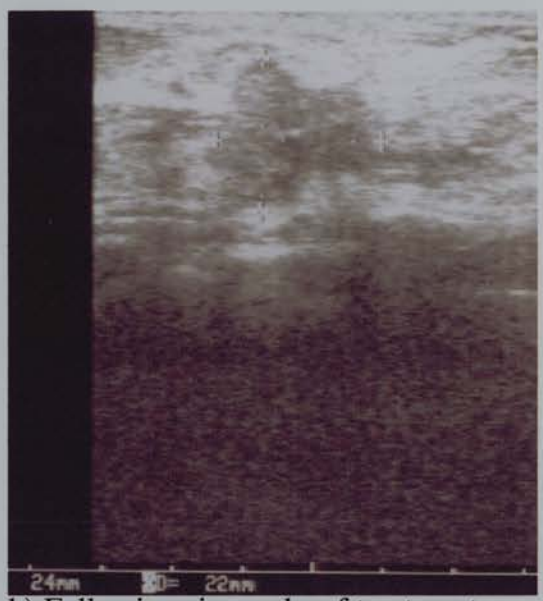
A responding tumour was defined as one in which there was a greater than twenty-five percent reduction in tumour volume at three months on ultrasound assessment (US). Those patients in whom clinical data alone was available (CLIN), response was defined as a greater than twenty-five percent reduction in clinical tumour volume. A similar definition was adopted in two patients assessed by mammography (MAMMO). Figure 6.2 illustrates mean serial reduction in tumour volume in non-responding and responding tumours. Mean tumour volumes, expressed as a percentage of the original tumour volume, were 97% in non-responding tumours at six weeks and 99% at 12 weeks. In responding tumours, corresponding values were 66.6% and 42%. Details of tumour volume measurements are described in the Appendix.

Fig 4.1.1. Serial ultrasound assessments of tumour volume, demonstrating reduced tumour volume during treatment with tamoxifen.

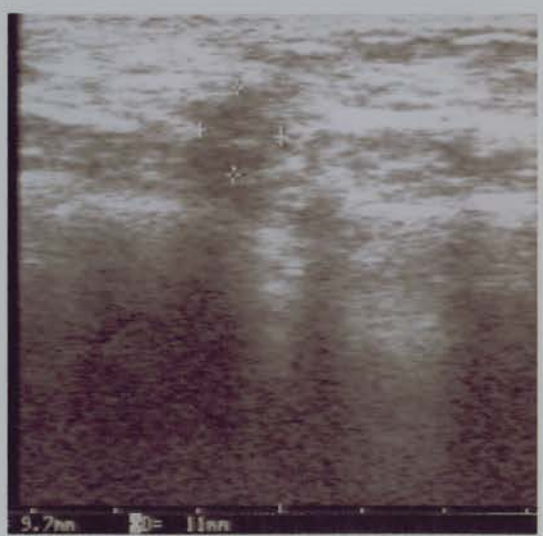
Tumour dimensions are shown ⊹



a) Following two weeks of treatment



b) Following six weeks of treatment



c) Following twelve weeks of treatment

Fig 4.1.2. Mammographic evidence of tumour response

a) Before treatment

b) After three months of tamoxifen treatment

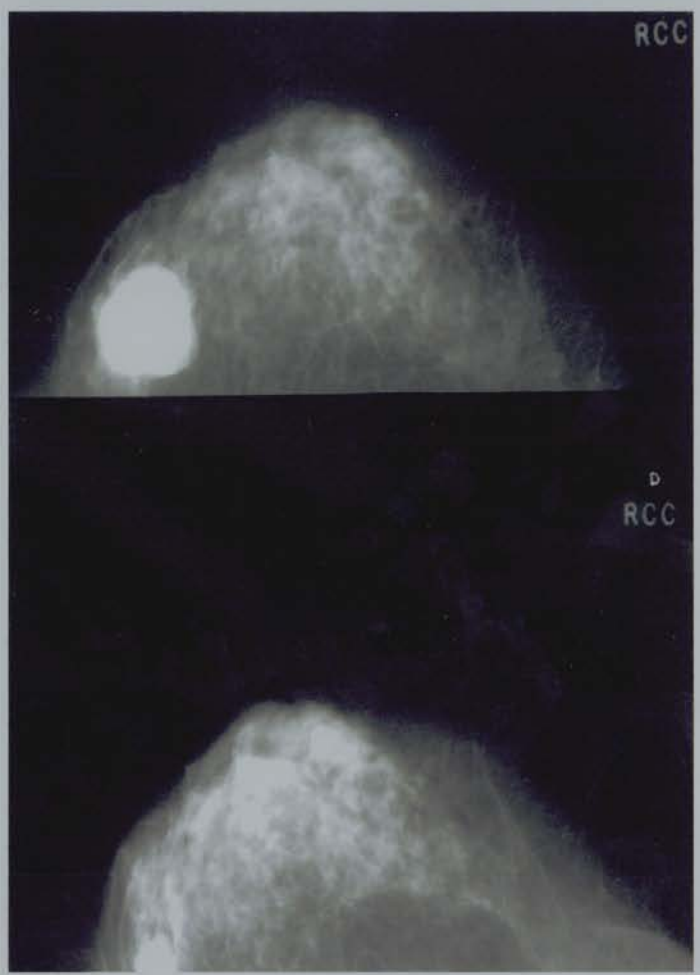


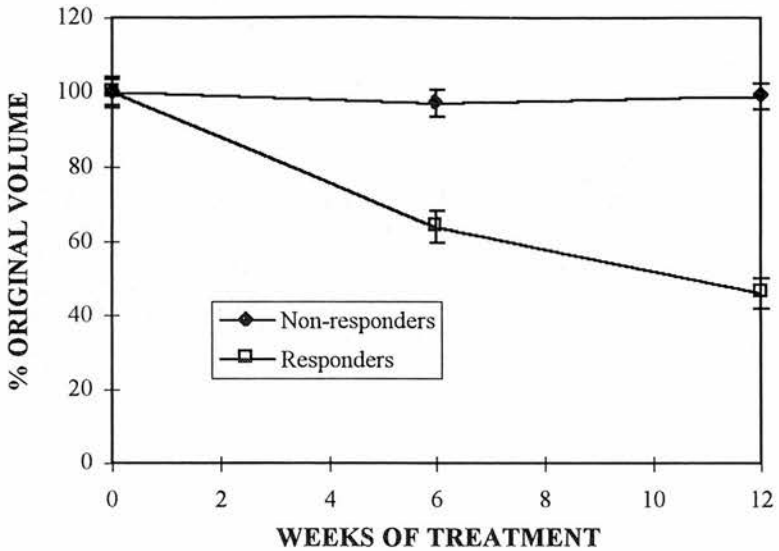
Table 4.1.1: Changes in relative tumour volume during treatment with tamoxifen.

Where tumour volumes are expressed at each time point as % original volume.

Non-responding tumours in **bold** type, NA=results not available.

Patient No.	Original volume	Tumour volume 1	Tumour volume 2	Mam. resp.	Assessment
615724L	**100**	**100**	**121**	**Decrease**	**US**
614905K	**100**	**100**	**86**	**Decrease**	**US**
296162H	**100**	**100**	**117**	**Increase**	**US**
143050M	**100**	**100**	**108**	**Reduction**	**US**
341122W	**100**	**100**	**81**	**NA**	**US**
624169K	**100**	**100**	**100**	**static**	**US**
558811X	**100**	**99**	**100**	**NA**	**US**
422318K	**100**	**100**	**100**	**increase**	**CLIN**
628483V	**100**	**100**	**115**	**static**	**US**
D077978R	**100**	**59**	**88**	**NA**	**US**
629111W	**100**	**100**	**92**	**static**	**US**
	100		**100**	**Static**	**MAMMO**
201525A	**100**	**100**	**100**	**Static**	**US**
625691A	**100**	**100**	**80**	**Reduction**	**US**
639991L	100	97	75	NA	CLIN
120288B	100	89	64	Static	US
612031H	100	60	23	NA	CLIN
614905K	100	27	22	Decrease	US
631385B	100	40	41	Decrease.	US
628989R	100	100	37	reduction	US
495945A	100	83	45	reduction	US
623397B	100	53	59	reduction	US
561829A	100	52	58		US
645382X	100	100	50		US
622149X	100	100	23	reduction	US
D345548V	100	100	42	reduction	US
614975H	100	50	58	reduction	US
640026R	100	74	34	static	US
639575E	100	100	100	reduction	MAMMO
638318X	100	73	51	static	US
637345V	100	54	41	reduction	US
637357K	100	44	64	static	US
84321A	100	44	35	reduction	CLIN
643001M	100	52	100	not seen	US
484933K	100	100	54	NA	US
266308W	100	26	25	Reduction	US
622926V	100	49	7	NANA	US
625368K	100	100	75	Static	US
D528581B	100	48	54	NA	US
10046V	100	23	20	Reduction	US
629740R	100	25	13	Reduction	US
626230E	100	39	25	NA	US

Figure 4.1.3: Mean serial changes in tumour volume with tamoxifen treatment in non-responding and responding tumours.
(Bars indicate standard error of mean)



Overall numbers of patients with non-responding and responding tumours are outlined in Table 4.1.2, demonstrating an overall response rate of 67%.

Table 4.1.2: Number of patients defined by response to tamoxifen:

RESPONDERS	28 (67%)
NON-RESPONDERS	14 (33%)
TOTAL	42 (100%)

4.2. Microvessel counts following staining with antibody to Factor VIII.

Core biopsies of tumour were taken before and following two weeks of treatment with tamoxifen. Tumour was available following treatment for three months in the form of repeat biopsies or surgical excision. Fifty-five patients were recruited into the study: of these, 10 patients refused second biopsies and three died during the course of treatment. Thus 42 patients completed the study. Assessments of tumour vascularity were made by performing microvessel counts (mvc) in three areas of high vascularity in all tumours before, during and after three month' treatment with tamoxifen. Endothelial cell staining was performed using immunohistochemical techniques as previously described. Sequential tumour sections were stained with two endothelial cell antibodies: antibody to Factor VIII (von Willebrand factor) and antibody to CD31. Results obtained using each antibody will be described separately. In addition, a subset of nine patients underwent serial monitoring with colour Doppler ultrasound, providing further information on change in tumour vascularity. Microvessel counts following staining with antibody to Factor VIII will be considered first.

Ranges of microvessel count during tamoxifen treatment in all patients are illustrated on Figure 4.2.1. The range of pre-treatment mvc was 6-25 (median: 14, mean: 14.5), of peri-treatment mvc was 6-22 (median: 15.5, mean: 15.2) and of post-treatment mvc was 0-23 (median: 15.5, mean: 15.1). Data are summarised on Table 4.2.1.

Figure 4.2.1: Changes in microvessel counts with tamoxifen treatment in all patients.

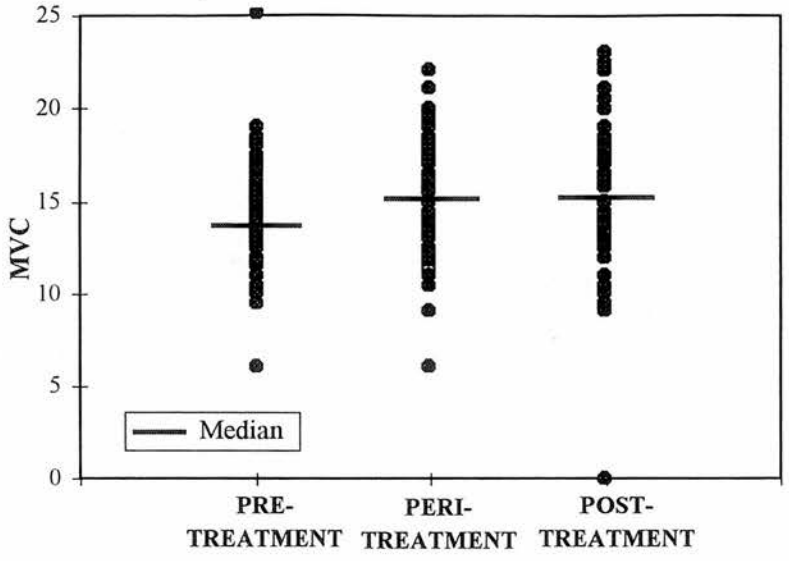


Table 4.2.1: Summary of data of microvessel counts following Factor VIII staining:

	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
Range	6-25	6-22	0-23
Median	14	15.5	15.5
Mean	14.5	15.2	15.1
Standard deviation	3.07	3.33	4.26
Standard error	0.47	0.51	0.66

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment mvc: p=0.40

Pre- v. post-treatment mvc : p=0.31

Per- v. post-treatment mvc p=0.90

Microvessel counts and response.

Pre-treatment mvc: Pre-treatment mvc were compared in non-responding (n=14) and responding (n=28) tumours as shown in Figure 4.2.2, illustrating no significant difference between the two groups. Pre-treatment mvc in non-responding tumours ranged from 6-17 (median: 13.5, mean: 13.3) and in responding tumours from 10-25 (median: 14.5, mean: 15.1). Data are summarised on Table 4.2.2.

Figure 4.2.2: Comparison of pre-treatment mvc in non-responding and responding tumours.

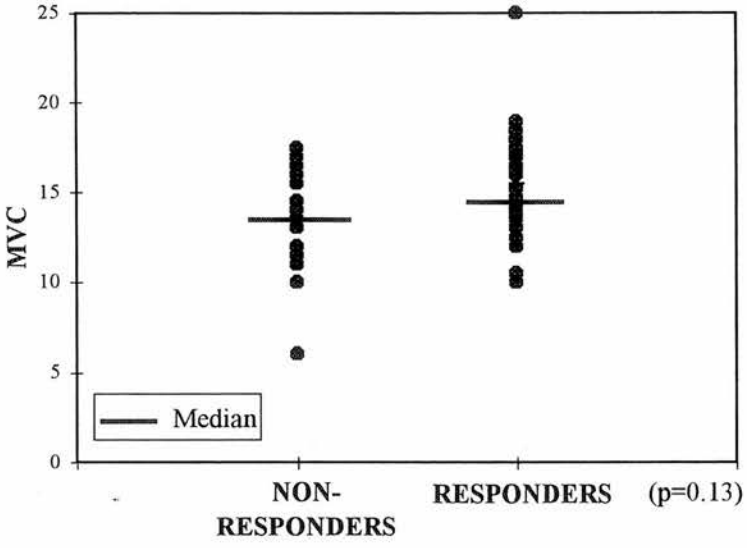


Table 4.2.2: Summary of pre-treatment mvc data:

	Pre-treatment mvc in non-responding tumours	Pre-treatment mvc in responding tumours.
Range	6-17	10-25
Median	13.5	14.5
Mean	13.3	15.1
Standard deviation	3.12	2.92
Standard error	0.84	0.55

Mann-Whitney test: p=0.13

Peri-treatment mvc: Peri-treatment mvc in non-responding and responding tumours are illustrated in Figure 4.2.3, demonstrating no significant difference between the two groups (p=0.23). Peri-treatment mvc ranged from 12-22 in non-responding tumours (median: 16, mean: 16.1) and from 6-22 in responding tumours (median: 14, mean: 14.7). Data are summarised in Table 4.2.3.

Figure 4.2.3:Comparison of peri-treatment mvc in non-responding and responding tumours.

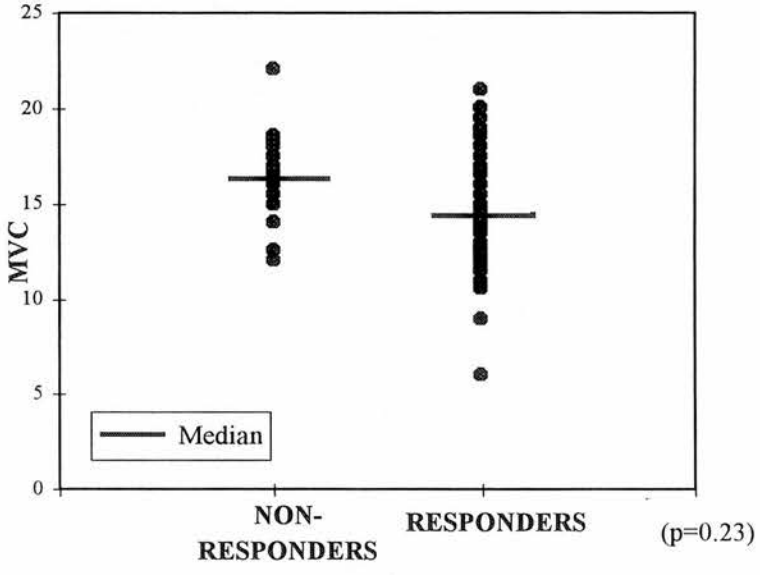


Table 4.2.3: Summary of peri-treatment mvc data:

	Peri-treatment mvc in non-responding tumours	Peri-treatment mvc in responding tumours.
Range	12-22	6-21
Median	16	14
Mean	16.1	14.7
Standard deviation	2.60	3.58
Standard error	0.69	0.68

Mann-Whitney test: p=0.23

Post-treatment mvc: Post-treatment mvc in non-responding and responding tumours are compared in Figure 4.2.4. Range of counts in non-responding tumours is 9-22 (median: 13.5, mean: 15.6) and in responding tumours 0-23 (median: 16, mean: 15.4). No significant difference between the two groups was demonstrated (p=0.31). Data are summarised on Table 4.2.4.

Figure 4.2.4: Comparison of post-treatment mvc in non-responding and responding tumours .

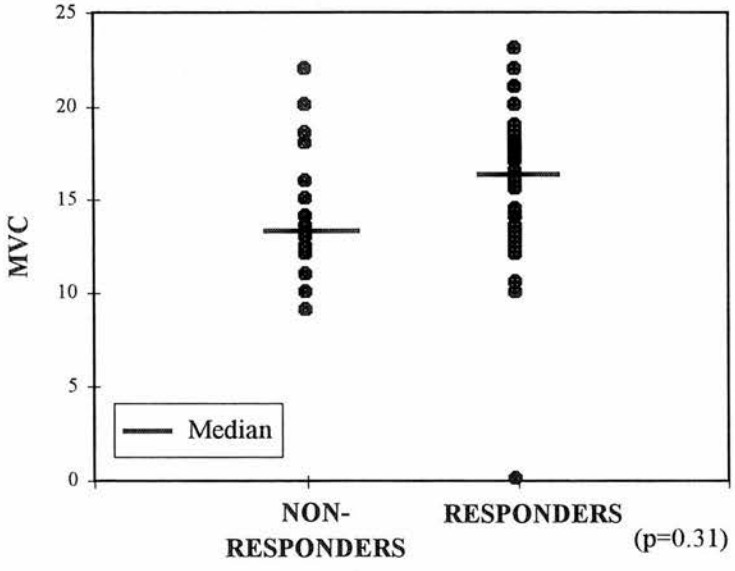


Table 4.2.4: Summary of post-treatment mvc data:

	Post-treatment mvc in non-responding tumours	Post-treatment mvc in responding tumours.
Range	9-22	0-23
Median	13.5	16
Mean	14.6	15.4
Standard deviation	3.82	4.51
Standard error	1.02	0.85

Mann-Whitney test: p=0.31

Changes in microvessel count in responding and non-responding tumours following treatment with tamoxifen.

Responding tumours: Microvessel counts in responding tumours taken before, during and after treatment with tamoxifen are compared in Figure 4.2.5. There was no significant difference in mvc between counts at the three time points. Data are summarised on Table 4.2.5.

Figure 4.2.5: Changes in mvc in responding tumours with tamoxifen treatment.

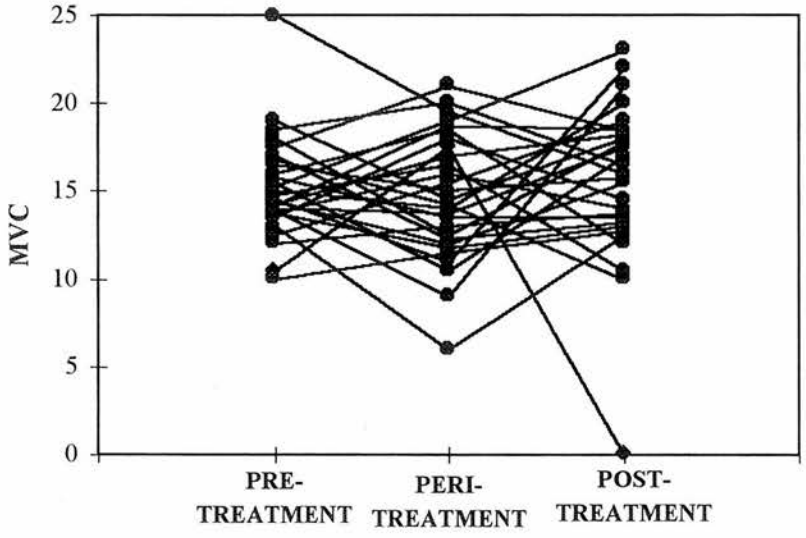


Table 4.2.5: Summary of data for responding tumours:

	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
Range	10-25	6-21	0-23
Median	14.5	14	16
Mean	15.1	14.7	15.4
Standard deviation	2.92	3.58	4.51
Standard error	0.55	0.68	0.85

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment mvc: p=0.56

Pre- v. post-treatment mvc: p=0.64

Peri- v. post-treatment mvc: p=0.34

Non-responding tumours: Changes in mvc in non-responding tumours are shown in Figure 4.2.6. There was a significant increase in mvc following 10-14 days of treatment (p=0.03). No significant difference between the counts taken before and after treatment was demonstrated (p=0.31) or between counts taken during and after treatment (p=0.33). Data are summarised on Table 4.2.6.

Figure 4.2.6: Changes in mvc in non-responding tumours with tamoxifen treatment.

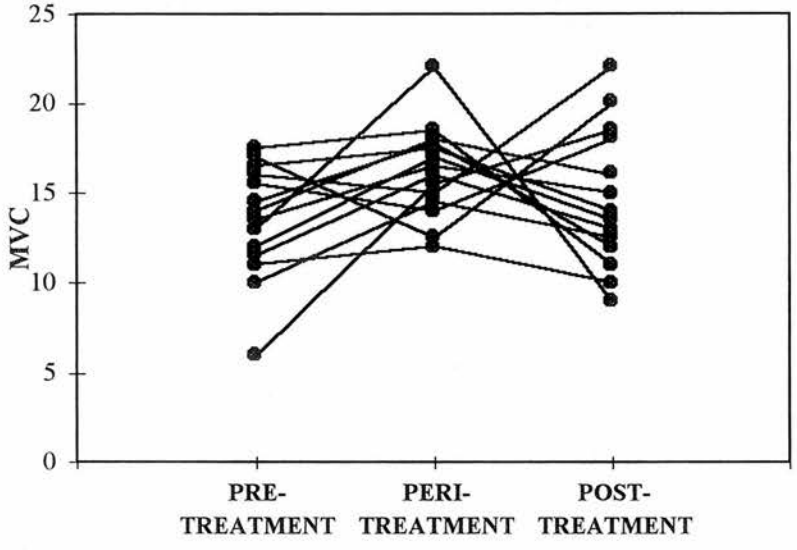


Table 4.2.6: Summary of data for non-responding tumours:

	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
Range	6-17	12-22	9-22
Median	13.5	16	13.5
Mean	13.3	16.1	14.6
Standard deviation	3.12	2.60	3.82
Standard error	0.83	0.69	1.02

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment mvc: p=0.03

Pre- v. post-treatment mvc: p=0.31

Peri- v. post-treatment mvc: p=0.33

Changes in microvessel counts with response to primary tamoxifen treatment.

The number of tumours in which mvc following staining with Factor VIII antibody increased, decreased or remained static was correlated with response and outlined on Tables 4.2.7a-c. In order to be defined as a change in mvc, the difference between counts had to be greater than 10%, which corresponded to the degree of inter-observer variation. Changes in mvc with treatment failed to reach statistical significance.

Table 4.2.7a: Change in mvc during first two weeks of treatment:
Pre- v. peri-treatment mvc.

	Increased mvc	Mvc static	Decreased mvc	TOTAL
Non-responders	8	4	2	**14**
Responders	13	5	10	**28**

Chi-squared test for trend: p=0.33

Table 4.2.7b: Change in mvc following three months' of tamoxifen treatment:
Pre- v. post-treatment mvc.

	Increased mvc	Mvc static	Decreased mvc	TOTAL
Non-responders	5	6	3	**14**
Responders	13	4	11	**28**

Chi-squared test for trend: p=0.11

Table 4.2.7c: Change in mvc between two weeks' and three months' tamoxifen treatment: Peri- v. post-treatment mvc.

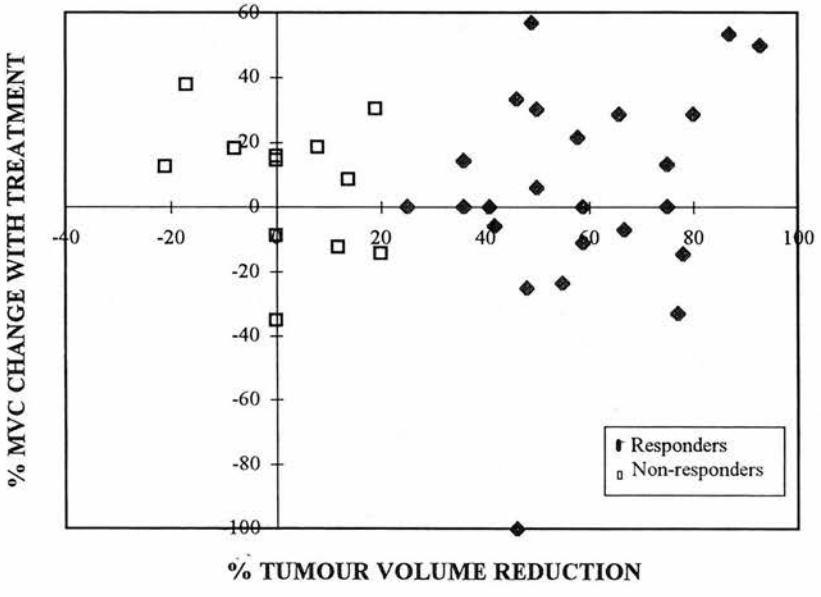
	Increased mvc	Mvc static	Decreased mvc	TOTAL
Non-responders	4	1	9	**14**
Responders	11	8	9	**28**

Chi-squared test for trend: p=0.10

Percentage reduction in tumour volume and change in microvessel count.

Ultrasound assessments of percentage reduction in tumour volume were available in 12 non-responding tumours (86%) and in 24 responding tumours (86%). The relationship between % reduction in tumour volume and degree of change in mvc with treatment is illustrated on Figure 4.2.7.

Figure 4.2.7: Relationship between degree of ultrasound response and % mvc change with treatment.



4.3. Microvessel counts following staining with antibody to CD31.

Ranges of microvessel counts in all patients irrespective of response are illustrated on Figure 4.3.1. The range of pre-treatment mvc was 8-22 (median: 14, mean: 14.9), peri-treatment mvc was 8-22 (median: 14, mean: 14.9) and post-treatment mvc was 0-23 (median: 15, mean: 14.8). There was no significant difference between the counts. Data are summarised on Table 4.3.1.

Figure 4.3.1: Changes in microvessel count with tamoxifen treatment in all patients.

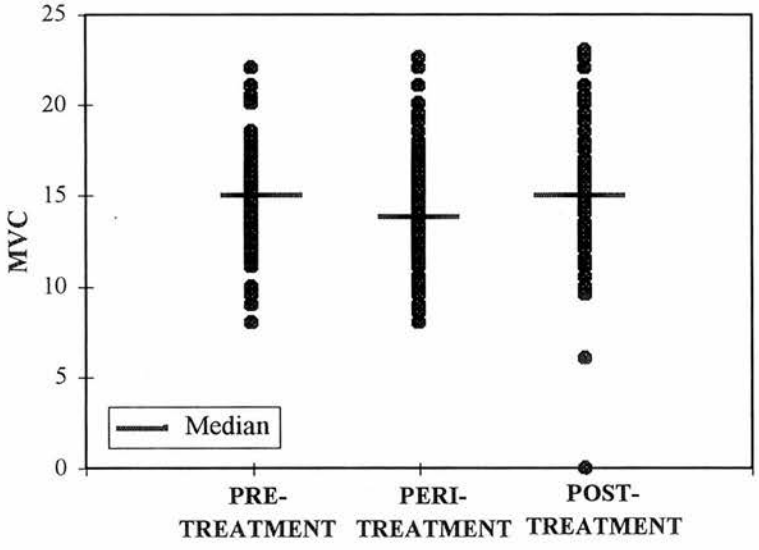


Table 4.3.1: Summary of data of microvessel counts following CD31 staining:

	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
Range	8-22	8-22	0-23
Median	15	14	15
Mean	14.9	14.4	14.8
Standard deviation	3.26	3.69	4.37
Standard error	0.50	0.57	0.67

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment mvc: p=0.26

Pre- v. peri-treatment mvc: p=1.0

Peri- v. post-treatment mvc: p=0.64

Microvessel counts and response.

Pre-treatment mvc: Pre-treatment microvessel counts (mvc) in non-responding and responding tumours are compared in Figure 4.3.2. Range of pre-treatment mvc in non-responding tumours was 9-18 (median: 14, mean: 14.1) and in responding tumours was 8-22 (median: 15, mean: 15.4). No significant difference between the two groups was detected (p=0.31). Corresponding data are summarised on Table 4.3.2.

Figure 4.3.2:Comparison of pre-treatment mvc in non-responding and responding tumours.

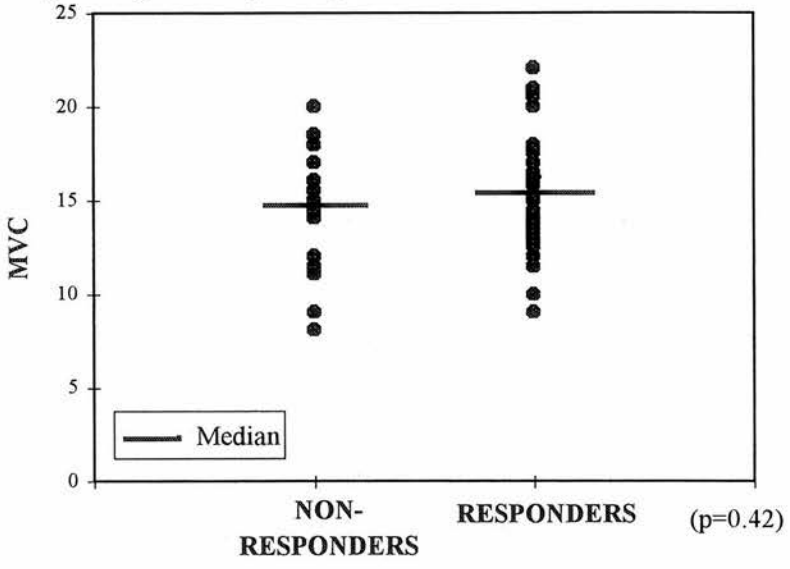


Table 4.3.2: Summary of pre-treatment mvc data:

	Pre-treatment mvc in non-responding tumours	Pre-treatment mvc in responding tumours
Range	8-20	9-22
Median	14.5	15
Mean	14.1	15.3
Standard deviation	3.57	3.09
Standard error	0.95	0.58

Mann-Whitney test: p=0.42

Peri-treatment mvc: Peri-treatment mvc, taken from core biopsies performed 10-14 days after commencement of tamoxifen therapy, are compared in Figure 4.3.3. The range of mvc in non-responding tumours was 9-20 (median: 14, mean: 14.2) and 8-22 in responding tumours (median: 14, mean: 14.5). There was no significant difference between the two groups. Data are summarised on Table 4.3.3.

Figure 4.3.3: Comparison of peri-treatment mvc in non-responding and responding tumours.

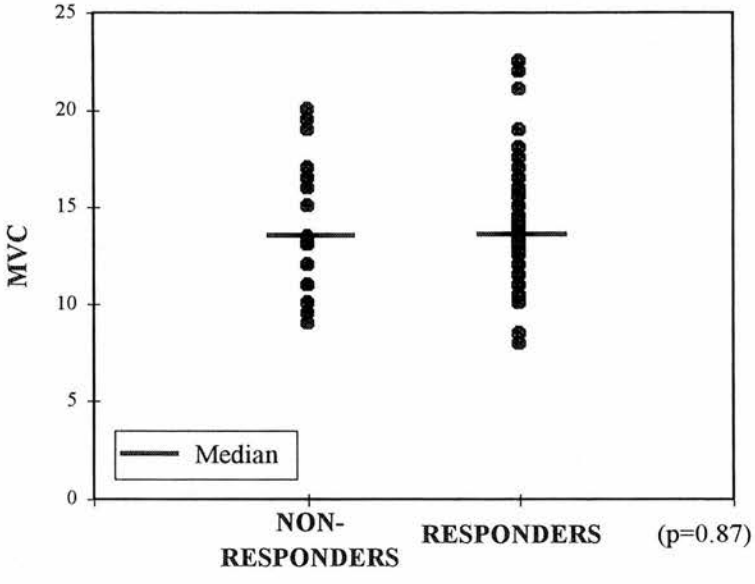


Table 4.3.3: Summary of peri-treatment mvc data:

	Peri-treatment mvc in non-responding tumours	Peri-treatment mvc in responding tumours
Range	9-20	8-22
Median	14	14
Mean	14.2	14.5
Standard deviation	3.77	3.72
Standard error	1.01	0.70

Mann-Whitney test: p=0.87

Post-treatment mvc: Post-treatment mvc in non-responding and responding tumours are compared in Figure 4.3.4. Range of mvc in non-responding tumours was 11-23 (median: 14.5, mean: 14) and in responding tumours 0-22 (median: 15, mean: 14.1). Figure 4.3.4 demonstrates no significant difference between the two groups (p=0.35). Data are summarised on Table 4.3.4.

Figure 4.3.4 : Comparison of post-treatment mvc in non-responding and responding tumours.

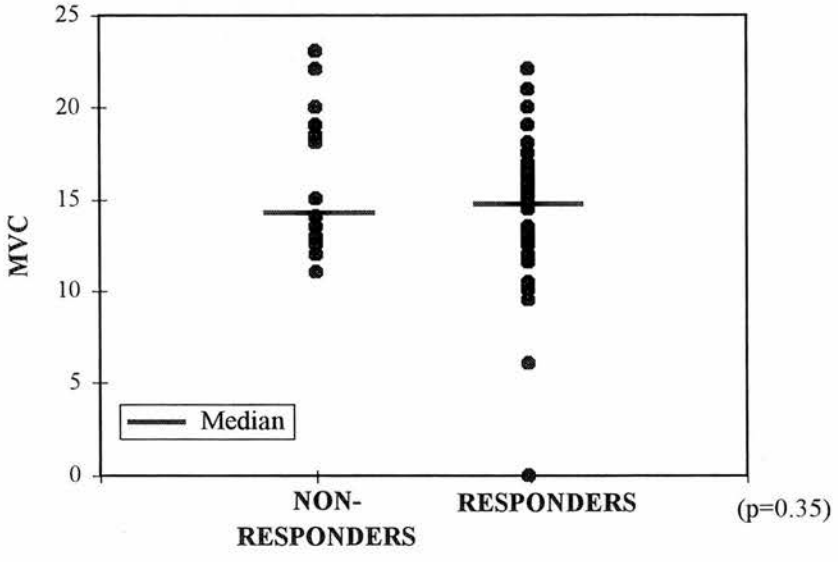


Table 4.3.4: Summary of post-treatment mvc data:

	Post-treatment mvc in non-responding tumours	Post-treatment mvc in responding tumours
Range	11-23	0-22
Median	14.5	15
Mean	16	14.1
Standard deviation	3.92	4.51
Standard error	1.05	0.85

Mann-Whitney test: p=0.35

Changes in microvessel count in responding and non-responding tumours during tamoxifen treatment.

Responding tumours: Microvessel counts in responding tumour sections taken before, during and after treatment with tamoxifen are compared in Figure 4.3.5. No significant trends in the difference between counts at each time point were identified. Data are summarised on Table 4.3.5.

Figure 4.3.5: Changes in mvc in responding tumours during tamoxifen treatment.

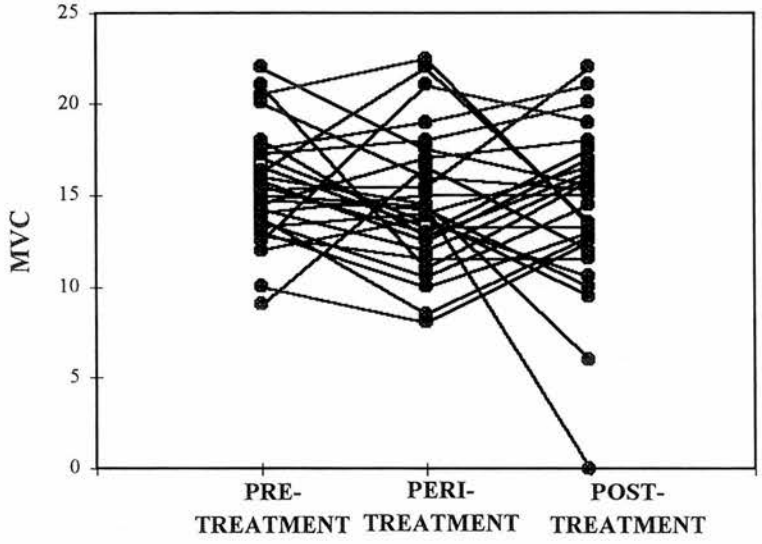


Table 4.3.5: Summary of mvc data in responding tumours:

	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
Range	9-22	8-22	0-22
Median	15	14	15
Mean	15.3	14.5	14.1
Standard deviation	3.09	3.72	4.51
Standard error	0.58	0.70	0.85

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment mvc: p=0.15

Pre- v. post-treatment mvc: p=0.39

Peri- v. post-treatment mvc: p=0.94

Non-responding tumours: Similar results were obtained in non-responding tumours, with no significant difference between counts at the three time points, illustrated on Figure 4.3.6. Data are summarised on Table 4.3.6.

Figure 4.3.6: Changes in mvc in non-responding tumours during tamoxifen treatment.

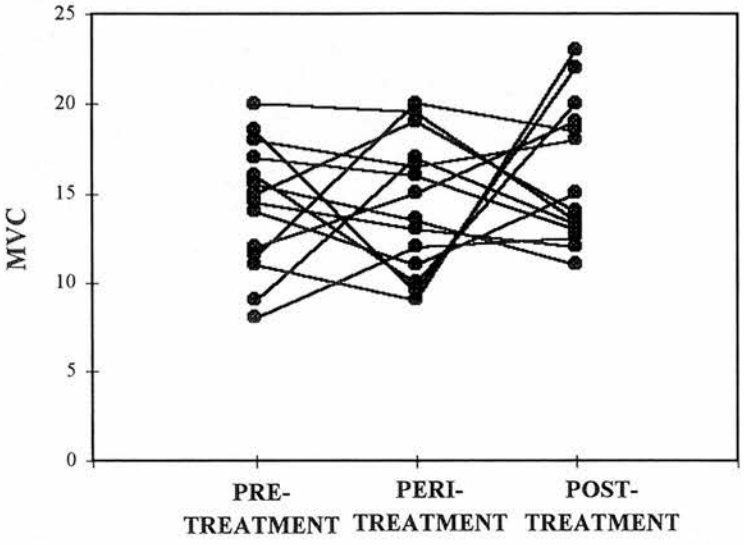


Table 4.3.6: Summary of mvc data in non-responding tumours:

	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
Range	8-20	9-20	11-23
Median	14.5	14	14.5
Mean	14.1	14.2	16
Standard deviation	3.57	3.77	3.92
Standard error	-0.95	1.00	1.05

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment mvc: p=1.00

Pre- v. post-treatment mvc: p=0.22

Peri- v. post-treatment mvc: p=0.64

Changes in microvessel counts with response to tamoxifen treatment.

The number of tumours in which mvc increased, decreased or remained static was correlated with response and is outlined on Tables 4.3.7a-c. No significant trends were identified.

Table 4.3.7a: Change in mvc during first two weeks of treatment:

Pre- v. peri-treatment mvc.

	Increased mvc	Mvc static	Decreased mvc	TOTAL
Non-responders	5	3	6	**14**
Responders	7	8	13	**28**

Chi-squared test for trend: p=0.61

Table 4.3.7b: Change in mvc following three months' of tamoxifen treatment:

Pre- v. post-treatment mvc.

	Increased mvc	Mvc static	Decreased mvc	TOTAL
Non-responders	6	4	4	**14**
Responders	11	5	12	**28**

Chi-squared test for trend: p=0.54

Table 4.3.7c: Change in mvc between two weeks' and three months' tamoxifen treatment: Peri- v. post-treatment mvc.

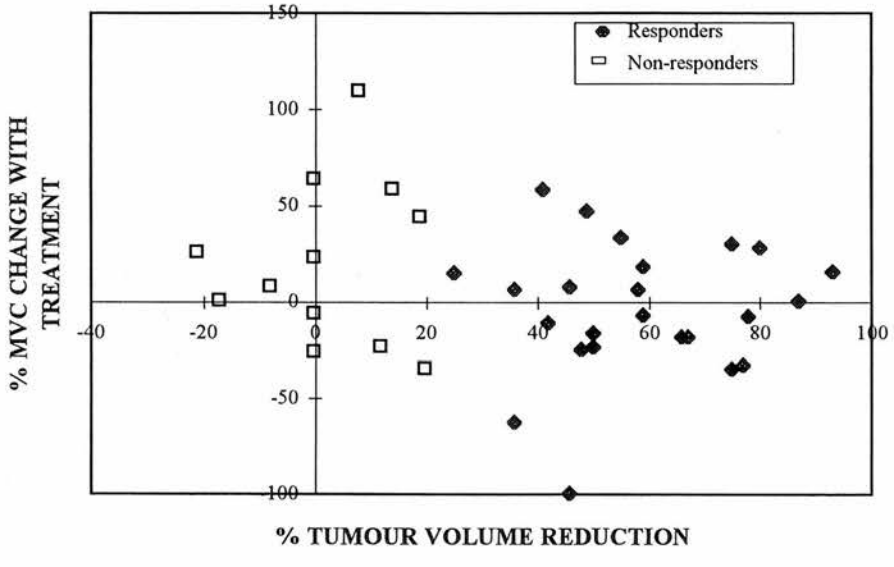
	Increased mvc	Mvc static	Decreased mvc	TOTAL
NON-RESPONDERS	7	1	6	**14**
RESPONDERS	11	7	10	**28**

Chi-squared test for trend: p=0.90

Percentage reduction in tumour volume and change in microvessel count.

The relationship between extent of tumour response and degree of change in mvc was investigated, which would identify any trends that may be apparent at extremes of response. The relationship is illustrated on Figure 4.3.7, and suggests similar ranges of mvc change in non-responding and responding tumours.

Figure 4.3.7: Relationship between degree of ultrasound response and % change in mvc with treatment.

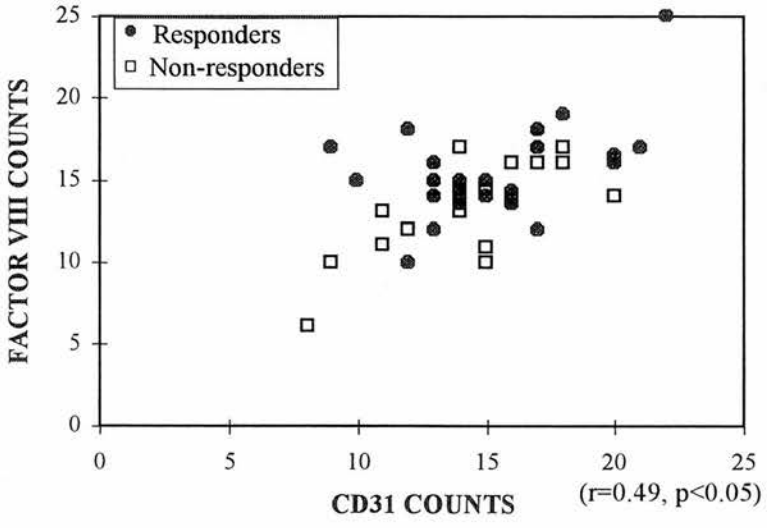


4.4 Comparison of microvessel counts performed following staining with antibodies to Factor VIII and CD31.

Having described changes in microvessel counts performed following staining with Factor VIII and CD31 antibodies separately, the following section will compare counts performed using each antibody. Figures 4.4.1-4.4.3 illustrate correlations between counts performed at each time point following staining with Factor VIII and CD31 antibodies in non-responding and responding tumours.

There was a significant correlation in pre-treatment mvc between the two antibodies in both non-responding and responding tumours, as illustrated in Figure 4.4.1.

Figure 4.4.1: Comparison of Factor VIII versus CD31 pre-treatment mvc in non-responding and responding tumours.



Spearman correlation coefficient for pre-treatment mvcs:

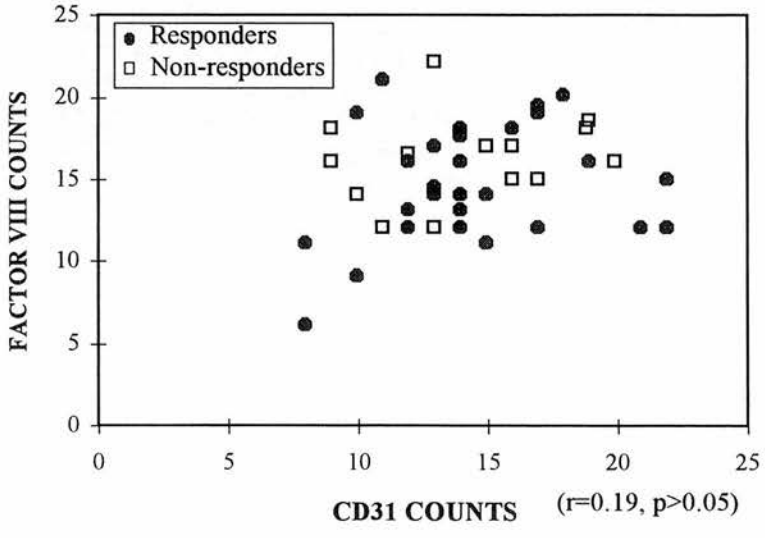
All tumours: r=0.49.

Non-responding tumours: r=0.78.

Responding tumours: r=0.44

There was a weaker correlation in Factor VIII versus CD31 counts during treatment, as shown on Figure 4.4.2, with a tendency for Factor VIII counts to be higher than CD31 counts.

Figure 4.4.2: Comparison of Factor VIII versus CD31 peri-treatment mvc in non-responding and responding tumours.



Spearman correlation coefficient for peri-treatment mvcs:

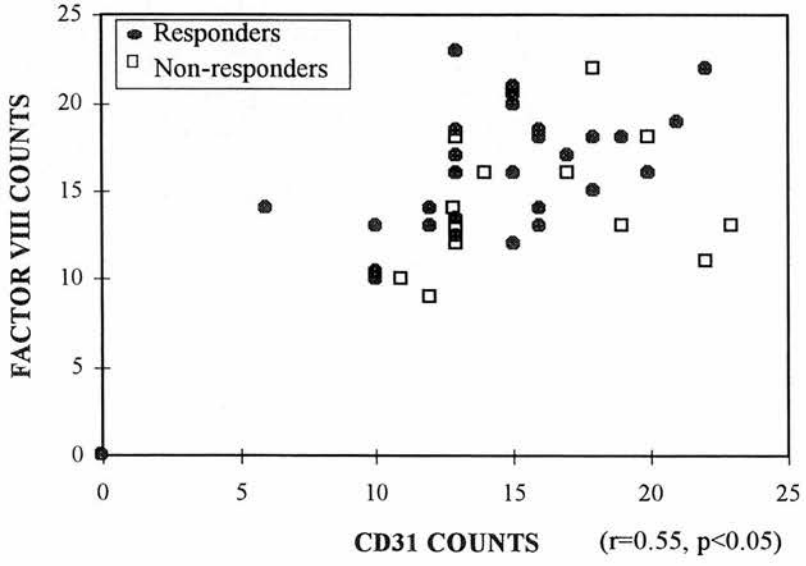
All tumours: r=0.19.

Non-responding tumours: r=0.20.

Responding tumours: r=0.21

A significant correlation was demonstrated between Factor VIII and CD31 counts after treatment in responding, but not non-responding tumours, as shown on Figure 4.4.3.

Figure 4.4.3: Comparison of Factor VIII versus CD31 post-treatment mvc in non-responding and responding tumours.



Spearman correlation coefficient for post-treatment mvcs:

All tumours: r=0.55.

Non-responding tumours: r=0.17.

Responding tumours: r=0.73.

4.5. Microvessel counts and other tumour parameters.

Further analyses were performed to determine whether correlations existed between microvessel counts and other tumour parameters which might affect tumour vascularity or response to tamoxifen treatment. Parameters investigated were lymph node involvement and level of oestrogen receptor level expression. These were correlated with mvc following staining with both Factor VIII and CD31 antibodies.

Lymph node involvement.

Nineteen patients underwent surgery at the end of three months of treatment with tamoxifen, the remainder did not have definitive surgery, but underwent repeat core biopsies for clinical reasons such as tumour inoperability, frail general medical condition or late response to tamoxifen warranting its continuation for longer than three months. Thus no information regarding axillary lymph node status was available in these patients. Nine patients with non-responding tumours (64%) and ten with responding tumours (36%) underwent axillary surgery. For the purpose of analysis, percentage of excised lymph nodes found to be positive was used to define lymph node involvement. Figure 4.5.1 illustrates the relationship between % lymph node involvement and response. The range of lymph node involvement in patients with non-responding tumours was 0-100% (median: 35, mean: 40.2) and in those with responding tumours was 0-61% (median: 6.25, mean: 16.7). Whilst this does suggest a trend for greater lymph node involvement in non-responding compared to responding tumours, this failed to reach statistical significance ($p=0.29$). Data are outlined on Table 4.5.1.

Figure 4.5.1: % lymph node involvement in patients with non-responding and responding tumours.

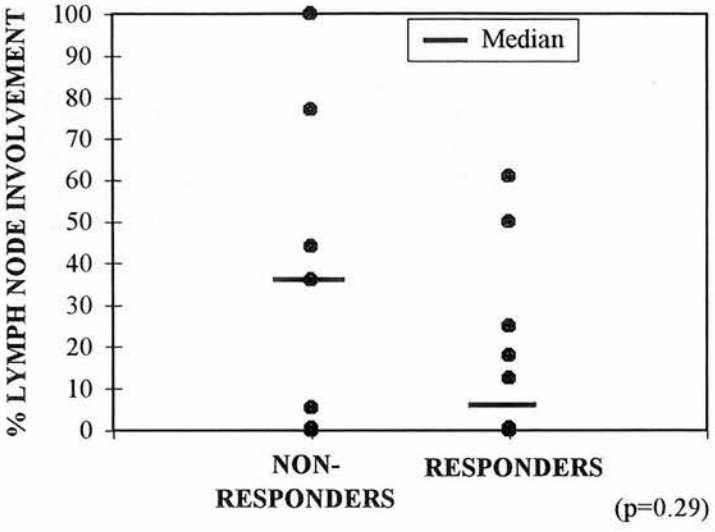


Table 4.5.1: Summary of data for % lymph node involvement and tumour response to tamoxifen:

	Non-responders	Responders
Range	0-100	0-61
Median	36	6.25
Mean	40.2	16.7
Standard deviation	42.8	22.5
Standard error	14.3	7.11

Mann-Whitney test: p=0.29

Lymph node involvement and microvessel counts following staining with antibody to Factor VIII.

Relationships between % lymph node involvement and mvcs following staining with Factor VIII antibody are illustrated on Figures 4.5.2-4.5.4. Relationships between % lymph node involvement and pre-treatment mvc, post-treatment mvc and % change in mvc with treatment were investigated. No significant correlation was found between the variables.

Figure 4.5.2: Relationship between % lymph node involvement and pre-treatment mvc.

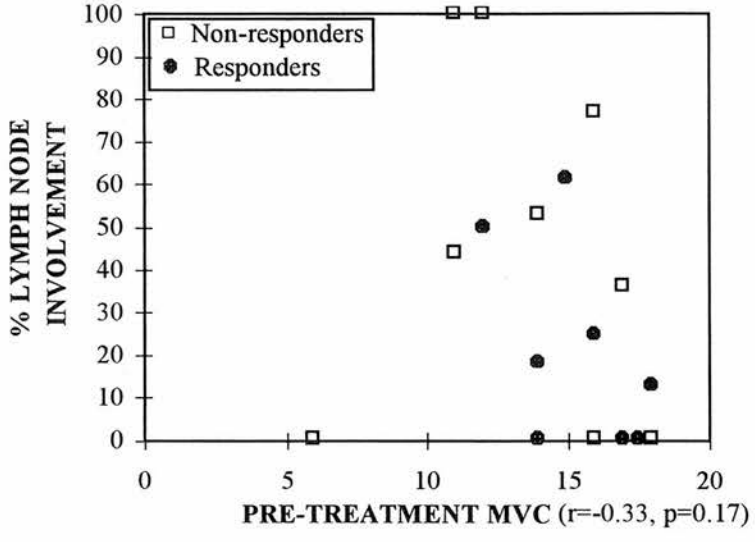


Figure 4.5.3: Relationship between % lymph node involvement and post-treatment mvc.

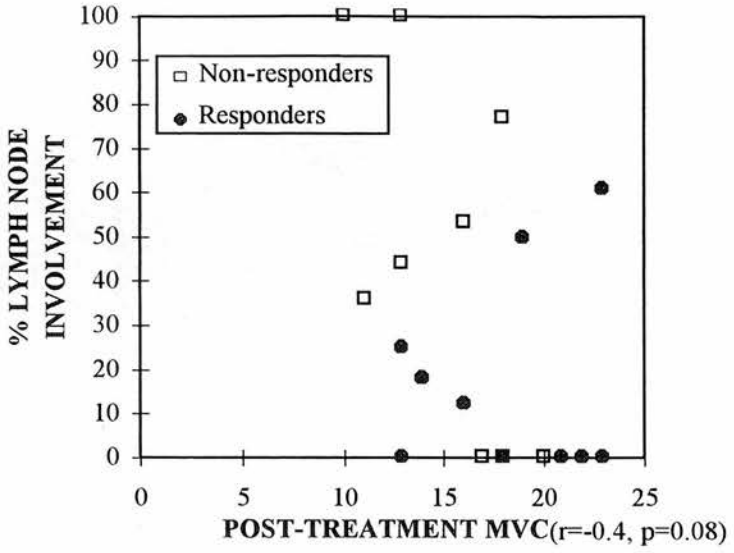
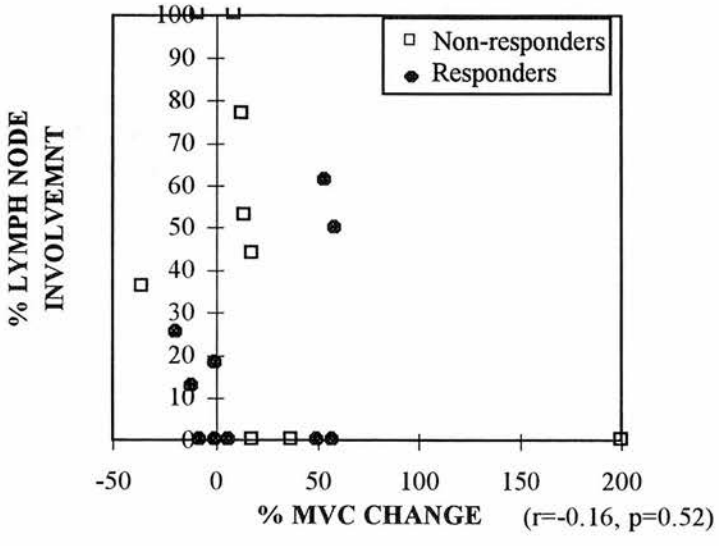


Figure 4.5.4: Relationship between % lymph node involvement and % mvc change with treatment.



Lymph node involvement and microvessel counts following staining with antibody to CD31:

Relationships between lymph node involvement and tumour vascularity assessments following staining with CD31 antibody are illustrated on Figures 4.5.5-4.5.7. No significant correlation was identified between microvessel counts and % lymph node involvement.

Figure 4.5.5: Relationship between % lymph node involvement and pre-treatment mvc.

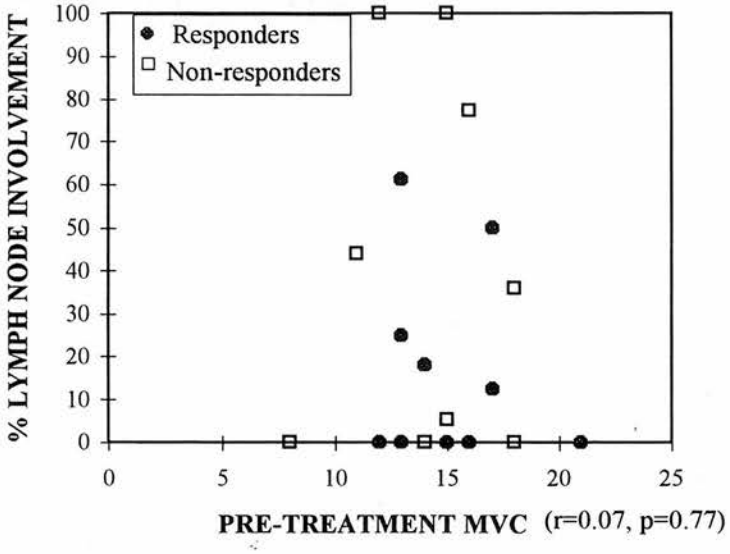


Figure 4.5.6: Relationship between % lymph node involvement and post-treatment mvc.

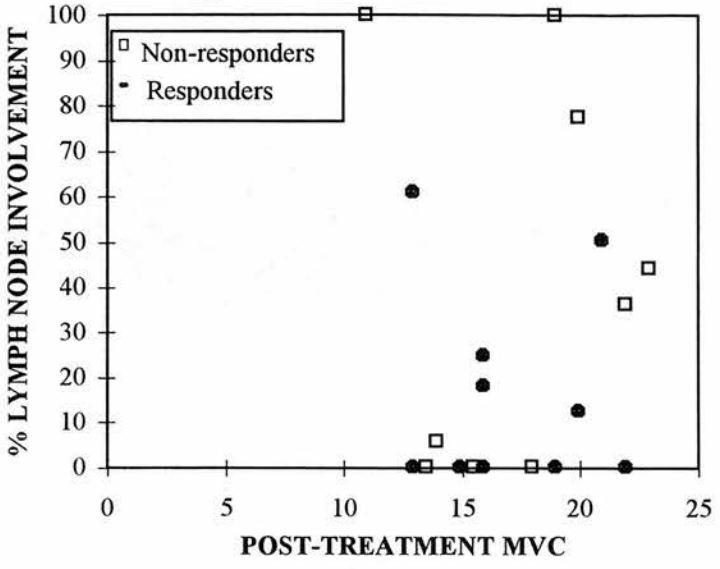
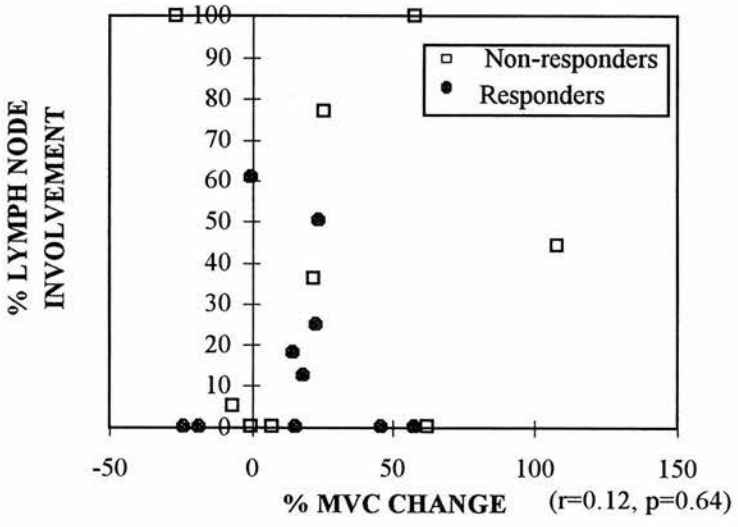


Figure 4.5.7: Relationship between % lymph node involvement and % change in mvc.



Level of oestrogen receptor expression.

Levels of oestrogen receptor (ER) expression assessed by immunohistochemistry were compared in non-responding and responding tumours, as is shown on Figure 4.5.8. The range of ER was 165-300 in non-responding tumours (mean: 259, median: 270) and 120-300 in responding tumours (mean: 268, median: 280). There was no significant difference in level of ER expression between the two groups (p=0.67). Data are summarised on Table 4.5.2.

Figure 4.5.8: Level of ER expression in non-responding and responding tumours.

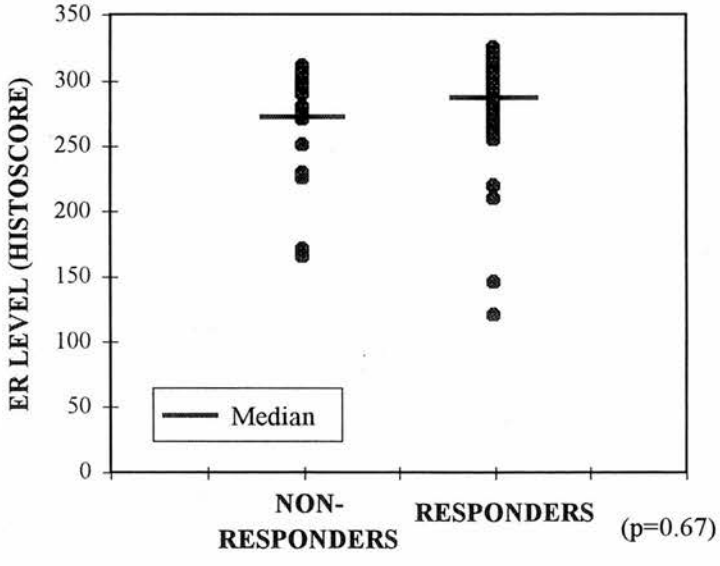


Table 4.5.2: Summary of data of ER and response:

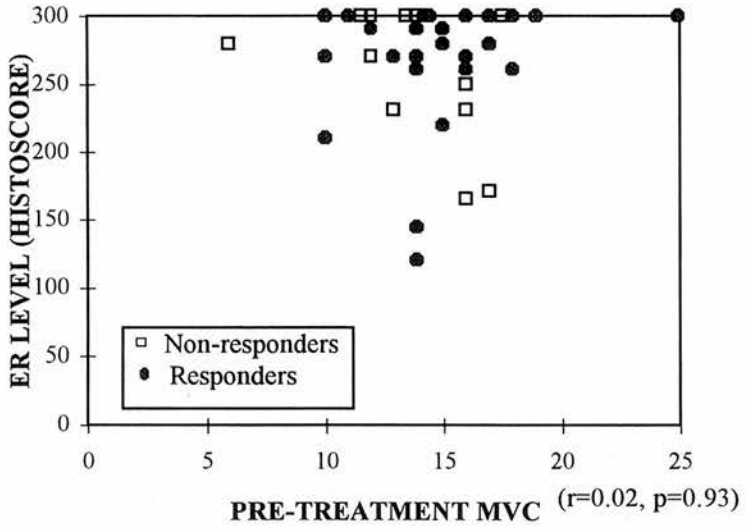
	Non-responders	Responders
Range	165-300	120-300
Median	270	280
Mean	259	268
Standard deviation	48.0	44.8
Standard error	13.3	8.46

Mann-Whitney: p=0.67

Level of ER expression and microvessel counts following staining with antibody to Factor VIII.

The relationship between level of ER expression, and pre-treatment mvc following staining with Factor VIII antibody, which were both determined on pre-treatment biopsies is illustrated on Figure 4.5.9, demonstrating no significant correlation between the two variables (r=0.02, p=0.93).

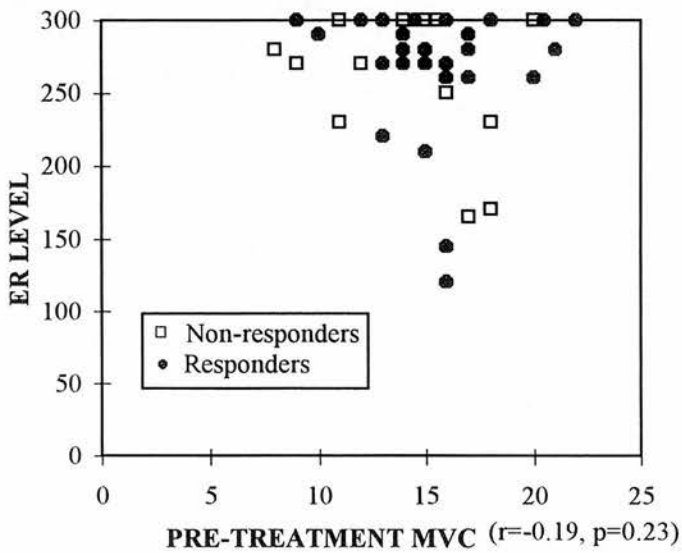
Figure 4.5.9: Relationship between level of ER expression and pre-treatment mvc.



Level of ER expression and microvessel counts: (CD31 staining)

The relationship between level of ER expression, and pre-treatment mvc following staining with CD31 antibody is illustrated on Figure 4.5.10, demonstrating no significant correlation between the two variables (r=0.19, p=0.23).

Figure 4.5.10: Relationship between level of ER expression and pre-treatment mvc.



4.6 Colour Doppler Ultrasound and assessment of tumour vascularity.

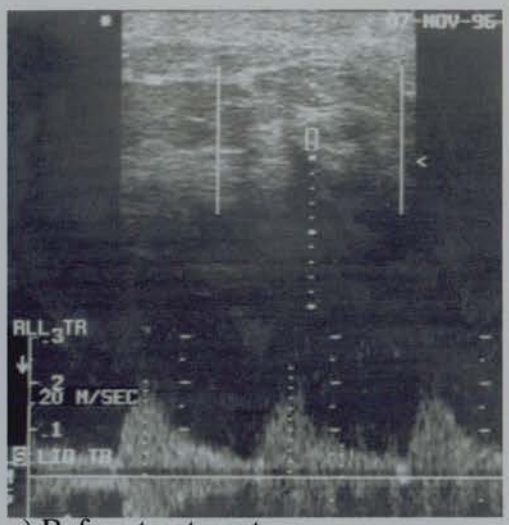
Colour Doppler ultrasound may provide a non-invasive method of assessing changes in tumour vascularity. Measurements of maximum systolic velocity (max. sys. vel.) in vessels feeding the tumours were made in nine patients. Scans were performed by one of two consultant radiologists at the time of diagnosis and immediately prior to the second core biopsy between ten and fourteen days later. In one patient a scan was repeated at completion of three months' treatment. Changes in maximum systolic velocity with response are illustrated on Figure 4.6.1. Data are outlined on Table 4.6.1.

Seven of the nine patients had tumours that failed to respond to tamoxifen: in three non-responders, tumours were negative for Colour Doppler signal, in two tumours there was an increase in maximum systolic velocity with treatment, velocity remained static in one tumour and decreased in one. Two responding tumours were assessed: in one tumour, there was a reduction in maximum systolic velocity observed only after three months of treatment, the other was negative for colour Doppler signal at diagnosis.

Table 4.6.1: Data for nine patients who underwent colour Doppler ultrasound of tumours:

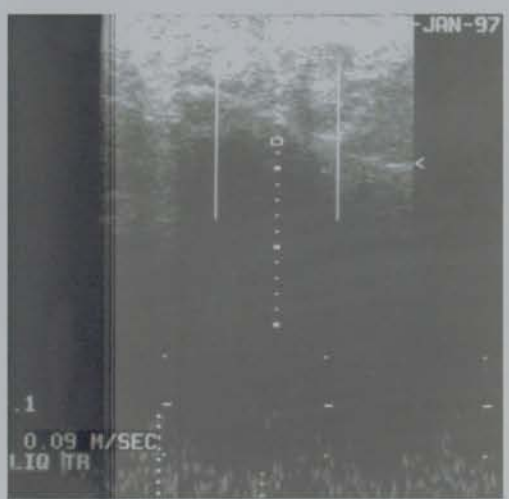
Patient no.	Date of scans	Tumour volume	CD signal +/-	max sys vel	Response
614905K	71196	22x24	1	0.17	
	281196	16x11x17	1	0.17	
	90197	14x11x17	1	0.09	R
296162H	120997	28x26x24	1	0.21	
	141097	28x26x24	1	0.21	NR
341122W	290597	28x25x30	1	0.42	
	180697	29x13x23	1	0.26	NR
558811X	120597	33x17x34	0		NR
628483V	150797	18x24x17	1	0.1	
	290797	18x24x17	1	0.11	NR
629111W	150797	28x18x29	0		NR
625368K	100797	12x15x16	1	0.1	
	310797	20x15x11	0		R
201525A	190198	26	0		NR
625691A	120597	32x12x33	1	0.07	
	180697	18	1	0.1	NR

Fig 4.6.1. Reduction in colour doppler ultrasound assessments of maximum systolic velocity (max.syst.vel) in a responding tumour during treatment with tamoxifen



a) Before treatment
max.syst.vel = 02m/s

b) Following six weeks of treatment
max.syst.vel = 0.15m/s



c) following twelve weeks of treatment max.syst.vel = 0.09m/s

4.7. Discussion.

Having demonstrated early changes in tumour vascularity in response to tamoxifen treatment in ER-positive breast cancer xenografts, a prospective study has been undertaken to determine whether such changes occurred in clinical tumours, thus providing potentially useful predictive information of response to primary treatment.

Forty-two patients with large breast cancers were treated with tamoxifen (20mgs daily) for a period of three months. Response to tamoxifen was defined as greater than 25% reduction in tumour volume on ultrasound or clinical measurements at three months. Twenty-eight patients had tumours that responded to tamoxifen according to this definition, giving a response rate of 67%. Tumour biopsies were performed at diagnosis and following two weeks of treatment. Patients underwent definitive surgery or repeat core biopsies at the end of three months of treatment.

Having stained tumour sections from each of the three time points with antibodies to Factor VIII and CD31, microvessel counts (mvc) were performed. When counts were correlated with response no significant difference was detected in pre-treatment mvc between non-responding and responding tumours. Peri-treatment mvc was significantly higher than pre-treatment counts in non-responding tumours following staining with antibody to Factor VIII ($p=0.03$), but not CD31. No overall change in mvc was detected following three months' treatment in either non-responding or responding tumours. In addition there was no difference in post-treatment counts between non-responding and responding tumours.

Counts performed following staining with Factor VIII and CD31 antibodies were compared. There was a significant correlation between counts before and after treatment in all tumours, but the relationship between counts during treatment failed to reach significance. When non-responding and responding tumours were analysed separately, peri-treatment mvc tended to be higher following staining with Factor VIII than CD31 in non-responding tumours. This was in keeping with changes observed during treatment, with a significant increase in mvc following two weeks of treatment in non-responders.

Two additional tumour parameters were examined in this study: lymph node involvement and level of oestrogen receptor (ER) expression. No correlation was identified with response in either parameter. In addition, there was no significant correlation between % lymph node

involvement and microvessel counts. In sections assessed following staining with Factor VIII antibody, there was a trend in the relationship between % lymph node involvement and post-treatment mvc, with those patients with higher post-treatment mvc more likely to have lymph node positive disease, but this failed to reach statistical significance ($p=0.09$). A similar, but significant trend was observed in the previous study. No significant correlation was identified between level of ER expression and pre-treatment mvc following staining with either CD31 or Factor VIII antibody.

The overall response rate of 67% was lower than that described in either the earlier study (75%) or in previous studies (Anderson *et al*, 1991). The definition of response as greater than 25% reduction in tumour volume at three months was unconventional. As discussed previously, UICC criteria define response as greater than 50% reduction in tumour volume for longer than one month, but such a definition was inappropriate for this study, in which the maximum period of treatment was three months. In the study described earlier in the thesis, patients with inoperable, T4 cancers were excluded and some patients were treated with tamoxifen for up to six months prior to surgery. Such inoperable cancers were included in this prospective study, which had a clear endpoint on completion of three months of treatment.

This study adopted novel methodology in terms of assessing tumour vascularity in sequential core biopsies during a primary course of tamoxifen treatment. Other studies have adopted the use of sequential fine needle aspiration for determining changes in hormone receptor level and proliferation (Makris *et al*, 1998), but assessment of tumour vascularity was not possible in such specimens. Previous work on angiogenesis assessment in primary breast cancer has focused on its role as a prognostic indicator. The majority of studies addressing the issue have found microvessel counts provided independent significant prognostic information on lymph node involvement, relapse-free and overall survival (Fox *et al*, 1995b; Gasparini *et al*, 1994; Weidner *et al*, 1992). The finding in the present study that there was no significant difference in pre-treatment mvc between non-responding and responding tumours is interesting in light of such studies. Response to tamoxifen has also been found to provide prognostic information (Cameron *et al*, 1997a). Pre-treatment mvc might thus have been expected to be higher in non-responding than responding tumours. However, it must be remembered that all patients selected for treatment with primary tamoxifen had tumours that were rich in oestrogen receptors, thus representing tumours with a good prognosis compared to ER-negative tumours (Thorpe & Rose, 1986).

In the present study, no significant changes in mvc were detected after treatment with tamoxifen for three months following staining with either Factor VIII or CD31 antibodies. This is in contrast to the findings of the study described earlier in the thesis, which demonstrated a significant reduction in mvc in responding tumours and an increase in non-responding tumours. The nature of pre-treatment biopsies was quite different in the two studies: the earlier study involved wedge biopsies, which contained a larger tumour volume than the core biopsies used in the present study. The reason for adopting the use of core biopsies in the present study is that they can be repeated during treatment with minimal discomfort to the patients.

Sequential core biopsies taken before, during and after a three month course of tamoxifen failed to demonstrate a reduction in mvc in responding tumours, as was described in the earlier study. There was a significant difference in mvc following two weeks' treatment with tamoxifen in tumour sections stained with antibody to Factor VIII, but not CD31 antibody. Peri-treatment mvc was higher than pre-treatment mvc in non-responding tumours ($p=0.03$). There was no difference between pre-and peri-treatment mvc in responding tumours. The difference was not maintained and post-treatment mvc was not significantly correlated with response. This is an interesting finding and worthy of comment. The pattern of change in tumour vascularity described in the first study was a reduction in responding tumours and an increase in non-responding tumours at completion of the period of treatment.

The failure of the present study to reproduce findings of the earlier study is likely to be related to methodological issues or to differences in the study populations. Methodological issues were addressed elsewhere in the thesis and demonstrated problems with reproducibility of counts performed in cores compared with tumour sections, taken either simultaneously or at separate time points with no intervening treatment. The technique of microvessel counting involved selection of areas of high vascularity in order to reduce the effect of tumour heterogeneity. In the present study, a small volume of tumour was available for assessment, thus reducing the likelihood that such biopsies contained the areas of highest vascularity. This is reflected in a difference in range of counts between the two studies: a maximum count of 23 was obtained in the present study, compared with 35 in the earlier study.

In addition to these points, several quantitative studies have shown that, for many experimental tumours, blood flow is higher in the tumour periphery than in central regions (Endrich *et al*, 1979; Tozer *et al*, 1990). This is in keeping with findings of the xenograft study described previously, in which the pattern of greater vessel distribution at the periphery compared with centrally was clearly seen. A similar phenomenon has been described in other xenograft studies (Furman-Haran *et al*, 1994; Lindner & Borden, 1997). A study observing patterns of microvessel density in association with ductal carcinoma in-situ identified development of a clearly defined rim of neovascularization around the tumour prior to onset of angiogenesis (Guidi *et al*, 1994). This pattern of new vessels developing from the tumour periphery and feeding into the central tumour may provide an explanation for the failure of the present study to reproduce findings of the earlier retrospective study. In the retrospective study, mvc performed in wedge biopsies and tumour transverse sections were compared. Wedge biopsies contain both peripheral and central areas of tumour and are therefore likely to contain vascular 'hot spots'. In the present study, counts were performed on core biopsy specimens and compared with cross-sections of tumour. Core biopsies were taken using a biopsy gun placed percutaneously at the tumour edge and fired into the central area of tumour. By the nature of this process, core biopsies contained a small sample of the tumour periphery. Thus, comparison of mvc in vascular hot spots of the cores and sections are likely to vary, with a tendency for higher counts to be found in tumour sections, as was demonstrated previously. It may be that the established method of microvessel counting in vascular hot spots needs to be amended when performing counts in different breast cancer specimens and such discrepancies may be less apparent if counts were performed in random fields.

There was a significant increase in mvc at two weeks of treatment following staining with antibody to Factor VIII in non-responding tumours. Of 14 non-responders, mvc increased in 8, remained static in 4 and decreased in 2 patients. This finding is in keeping with the earlier study in which there was a significant increase in mvc in non-responding tumours at three months. Such findings are of concern in that they suggest that tamoxifen may actually be exerting a positive effect on tumour growth. This increase may be merely a reflection of growth of untreated tumours; although no such significant increase was found in counts performed on cores and subsequent sections taken with no intervening treatment, there was a trend for counts to be higher in later tumour sections.

The difference between counts obtained following staining with antibody to CD31 and Factor VIII is noteworthy. In an earlier chapter, the relationship between counts performed following staining with the two antibodies was investigated and demonstrated a good correlation between counts in both core biopsies and tumour sections. This was confirmed in the present chapter, in which Factor VIII and CD31 counts were compared at each time point. There was no significant correlation in counts following two weeks of treatment: indeed, comparison of peri-treatment mvc in non-responding tumours revealed a non-significant trend for Factor VIII counts to be higher than CD31 counts. This may be a reflection of the differing roles of the two antibodies and varying effects of tamoxifen on their expression. Such variation has not been previously described. Factor VIII antigen plays a role in platelet aggregation and adhesion (Fajardo, 1989); the antibody stains both blood and lymphatic vessels and, although staining does not include all microvessels, it is more reliable than other endothelial cell markers. CD31 plays a role in platelet adhesion during inflammatory processes and wound healing (Parums *et al*, 1990) and the antibody has been shown to give consistently higher counts than Factor VIII (Martin *et al*, 1997).

Information regarding lymph node status was available in a subgroup of 19 patients, who were treated by definitive surgery at the end of three months' tamoxifen. No significant correlation was found between lymph node involvement and response or microvessel counts. In the retrospective study previously described, a significant association was found between % lymph node involvement and both post-treatment mvc and % change in mvc. Patients in whom post-treatment mvc was high were more likely to have lymph node positive disease than patients with lower mvc. In this study, in sections stained with antibody to Factor VIII, a similar trend was seen which failed to reach statistical significance ($p=0.08$). This may reflect the small number of patients in whom such assessment was available.

Lack of association between ER level and response in the present study is contradictory to findings in previous studies (Gaskell *et al*, 1992; Osborne *et al*, 1980) in addition to that outlined earlier in the thesis. Standard method of assessment of ER has changed in the Edinburgh Breast Unit between the period of recruitment into the two studies and now involves immunohistochemical evaluation of tumour sections. At the time of the earlier study a biochemical method was adopted and this discrepancy may have led to the different results. In addition, assessment of ER in the present study was made on core biopsy specimens; in the previous study assessments were made on tissue from wedge biopsies. The problems of sampling which have been described in association with tumour vascularity

assessments in this thesis may exist in the assessment of other parameters including the oestrogen receptor. However one study addressing this issue described 100% concordance in ER asessment of cores and tumour sections when an immunohistochemical technique was adopted (Jacobs *et al*, 1998). The lack of association between ER level and pre-treatment mvc was similar in this study to that described previously in the thesis.

It may be that discrepancies in findings of the present study compared with the earlier study are related to differences in the study populations in addition to the methodological variations that have been discussed. In the present study, patients with large inoperable T4 were included, in contrast to the earlier study. The lower age limit in the earlier study was 70 years, in the present study patients had to be postmenopausal. In addition patients were included in the study for three months only, but in the earlier study they may have been treated for up to six months. These differences may contribute to the discrepancies in mvc assessments and response and the failure in this study to identify an association between ER expression and response to tamoxifen.

Other parameters may provide useful information for predicting early response to treatment. It is possible that other factors relating to angiogenesis may be of use, such as vascular endothelial growth factor (VEGF) expression, which is expressed in normal cells with increased expression in some tumours, including breast cancers (Toi *et al*, 1995). Levels of VEGF expression have been found to provide significant prognostic information in primary breast cancers (Gasparini *et al*, 1997). Alternative, non-invasive methods of assessing tumour angiogenesis are under development. A study investigating the relationship between mvc and Doppler flow parameters failed to demonstrate a significant correlation between the two (Peters-Engl *et al*, 1998). The authors suggested that the two investigations assess different aspects of the vasculature: mvc provides information on the microvasculature, Doppler signals on the macrovasculature (Peters-Engl *et al*, 1998). The number of patients in whom serial colour Doppler ultrasound was performed in this thesis is too small to permit meaningful comment: of nine patients assessed, three had tumours that were colour Doppler-negative. In the remaining four non-responding tumours, there was an increased maximum systolic velocity in two. Of two responding tumours, one tumour was rendered colour Doppler-negative following two weeks of treatment, in the other a reduction in velocity was observed later. These findings are of interest, suggesting a possible role for colour Doppler ultrasound in monitoring response to treatment and this warrants further investigation. Another non-invasive method of angiogenesis assessment currently under investigation is

contrast-enhanced magnetic resonance imaging (MRI). MR enhancement has been found to be associated with high vessel densities, but data showed considerable variation and therefore was not considered to be an accurate predictor of microvessel density (Buckley *et al*, 1997; Stomper *et al*, 1997). In another study, MRI and a contrast agent directly targeting the endothelial integrin $\alpha V\beta 3$ provided enhanced and detailed imaging of rabbit carcinomas, including identification of angiogenic hot spots, which had not been previously detected by MRI (Sipkins *et al*, 1998). The development of new contrast agents and technological improvements in MRI may provide an important non-invasive method of assessing tumour angiogenesis. In addition, other parameters unrelated to angiogenesis may provide valuable information, as they may be less affected by tumour heterogeneity and by previous surgical manipulation of the tumour.

In summary, the use of sequential core biopsies during treatment with primary tamoxifen has failed to identify any clear relationship between change in tumour vascularity and response. In combination with results described in studies of methodological issues, it is likely that small tumour biopsies were not sufficiently accurate in reflecting overall tumour vascularity to be of value. Tumour heterogeneity poses a particular problem in the method of microvessel counting as this involves selection of the most vascular areas of tumour. Such areas are less likely to be present in small biopsies and it may be more appropriate to perform counts in random fields. In addition, angiogenesis is stimulated in response to injury and the effect of previous tumour manipulation may mask any early effect of tamoxifen. Alternative early markers of response to tamoxifen must be sought.

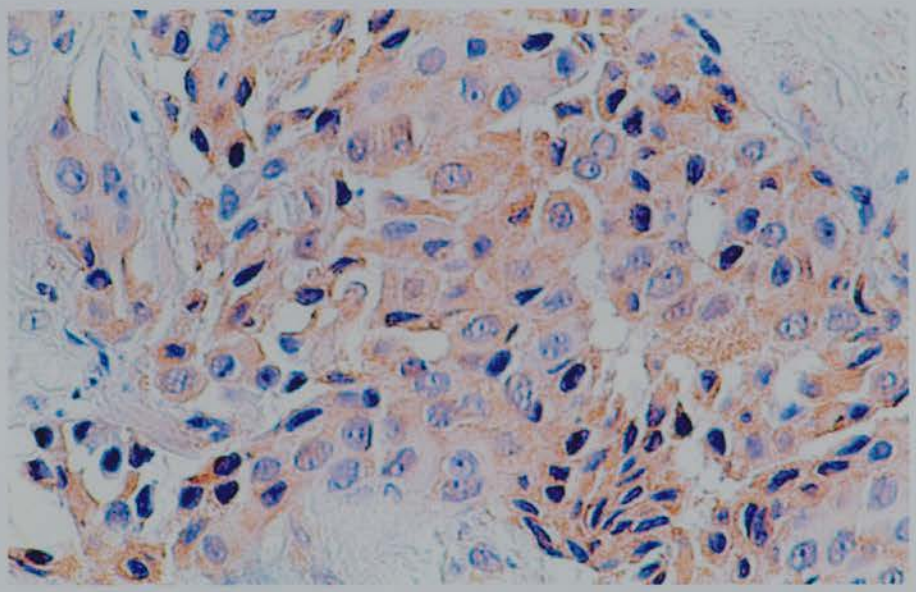
5. Changes in % tumour cells expressing vascular endothelial growth factor (VEGF) and tamoxifen treatment.

In this chapter % tumour cells expressing VEGF were compared before and after treatment with tamoxifen and correlated with response. The study involved assessment of VEGF expression in those tumours described in Results chapter 2 of this thesis. Staining with antibody to VEGF was performed in tumours from fifty-seven patients. In nine patients, staining with VEGF antibody failed despite successful staining of positive controls in the same run. These comprised sections cut from the oldest tumour blocks that had been stored for up to six years. Thus the total number of patients studied in this section was forty-eight: this comprised twelve patients with non-responding (25%) and thirty-six with responding tumours (75%) according to criteria detailed earlier. Following staining with antibody to VEGF, assessments were made of % tumour cells staining and intensity of staining as described in the methods section.

When assessments of intensity were performed it became apparent that there was marked variation in staining of sections of the same breast tumour used as positive controls between different batches of antibody, thus rendering such assessments inaccurate. It was, therefore, decided to score tumours according to % cells staining positive for VEGF only. The scoring system comprised a score of up to 4, where a score of 0 was given if <1% cells staining, 1 if 1-25% cells stained, 2 if 26-50% cells stained, 3 if 51-75% cells stained and 4 if >76% cells stained positively for VEGF. Scoring was performed on tumour cells only in three high power field (x400). The total of three scores gave the level of VEGF expression for that tumour. Thus the highest score was 12.

An example of staining with antibody to VEGF is illustrated in Figure 5.1, demonstrating 100% tumour cells staining positively.

Fig 5.1 Tumour staining with antibody to VEGF at high power (x400). Positively staining cells are highlighted in brown.



5.1 % VEGF-positive tumour cells and response.

Pre-treatment: All tumours except for two (responders) expressed VEGF in a percentage of tumour cells. There was no significant difference in % cells staining for VEGF between non-responding and responding tumours, as illustrated on Figure 5.1.1. Range of values in non-responding tumours was1-12 (median: 12, mean: 10.5) and in responding tumours was 1-12 (median: 12, mean: 9.56). Data are outlined on Table 5.1.1. Actual counts are detailed in the Appendix to this chapter.

Figure 5.1.1: Comparison of % tumour cells staining for VEGF in non-responding and responding tumours before treatment.

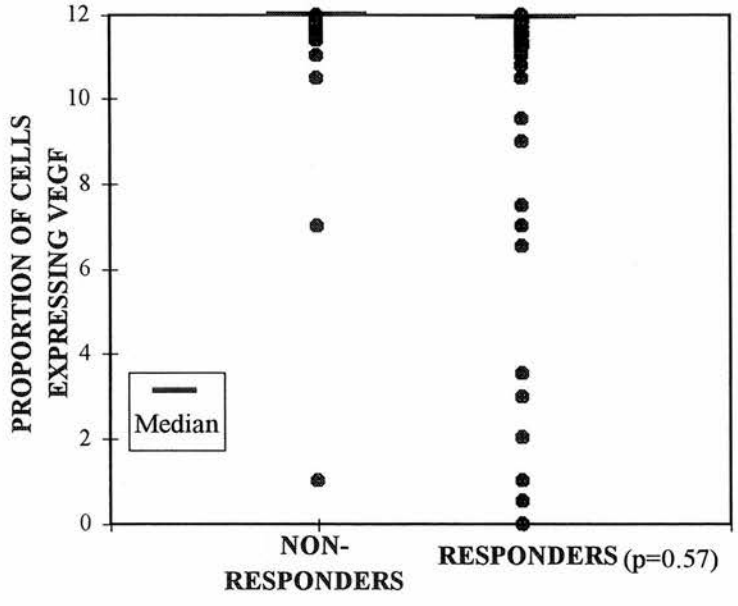


Table 5.1.1: Comparison of VEGF expression before treatment in non-responding and responding tumours.

	Non-responders	Responders
Range	1-12	0-12
Median	12	12
Mean	10.5	9.56
Standard error	0.96	0.66
Standard deviation	3.32	3.97

Mann-Whitney test: p=0.57

Post-treatment: There was a trend for a reduction in % of cells expressing VEGF in the total group of tumours following treatment, which was more marked in responding than non-responding tumours. There was, however, no significant difference in % cells expressing VEGF between non-responding and responding tumours, as illustrated on Figure 5.1.2. Range of post-treatment VEGF in non-responding tumours was 0-12 (median: 10, mean: 8.5). Range in responding tumours was 0-12 (median: 8, mean: 7.67). Data are outlined in Table 5.1.2.

Figure 5.1.2: Comparison of % tumour cells staining for VEGF in non-responding and responding tumours after treatment.

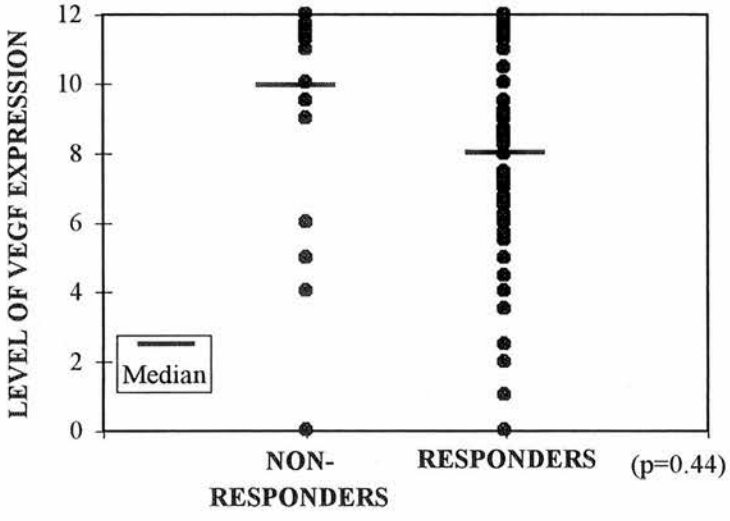


Table 5.1.2: Comparison of VEGF expression after treatment in non-responding and responding tumours.

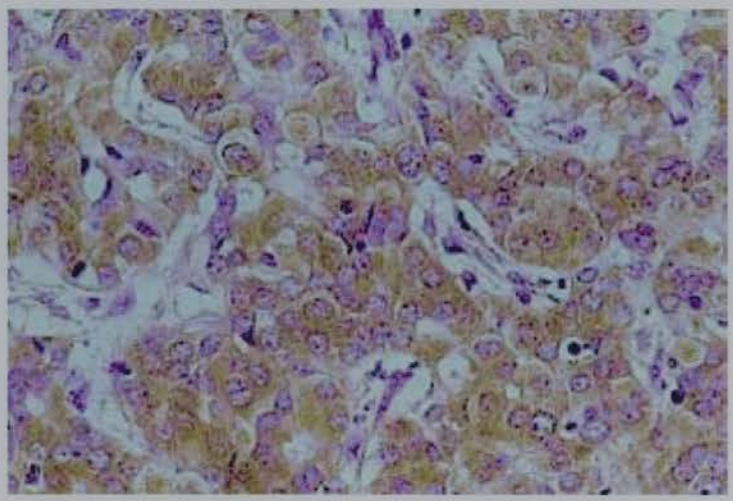
	Non-responders	Responders
Range	0-12	0-12
Median	10	8
Mean	8.5	7.67
Standard error	1.13	0.58
Standard deviation	3.90	3.45

Mann-Whitney test: p=0.44

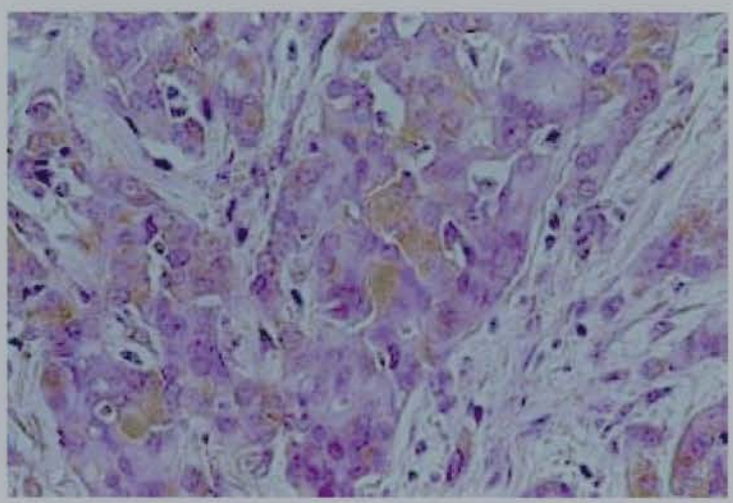
Changes in % VEGF-positive cells in non-responding and responding tumours following treatment with tamoxifen.

Changes in % cell staining for VEGF occurred in both non-responding and responding tumours. An example of reduced VEGF expression following treatment is illustrated in Figure 5.1.3.

Fig 5.1.3. Reduction in percentage cells staining for VEGF following treatment. Positively staining cells are highlighted in brown. Shown at high power (x400).



a) before treatment



b) after treatment

Responding tumours: The proportion of tumour cells expressing VEGF before and after treatment in responding tumours are illustrated in Figure 5.1.4, demonstrating a significant reduction in % cells staining following treatment. Data are outlined in Table 5.1.3.

Figure 5.1.4: Comparison of VEGF expression in responding tumours before and after treatment.

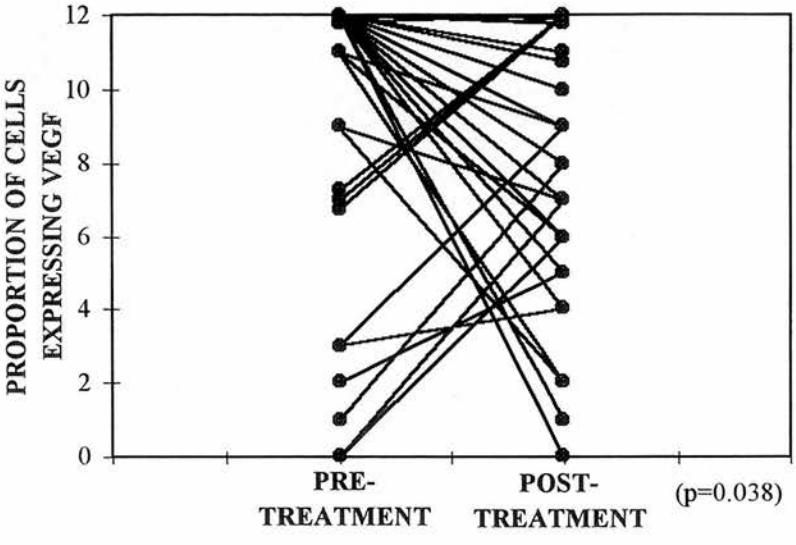


Table 5.1.3: Changes in proportion of cells expressing VEGF in responding tumours following treatment with tamoxifen:

	Pre-treatment	Post-treatment
Range	0-12	0-12
Median	12	8
Mean	9.6	7.67
Standard error	0.66	0.58
Standard deviation	3.98	3.45

Mann-Whitney test: p=0.04

Non-responding tumours: Comparison of level of VEGF expression in non-responding tumours before and after treatment with tamoxifen is illustrated in Figure 5.1.5; no significant difference was detected. Data are outlined on Table 5.1.4.

Figure 5.1.5: Comparison of VEGF expression in non-responding tumours before and after treatment.

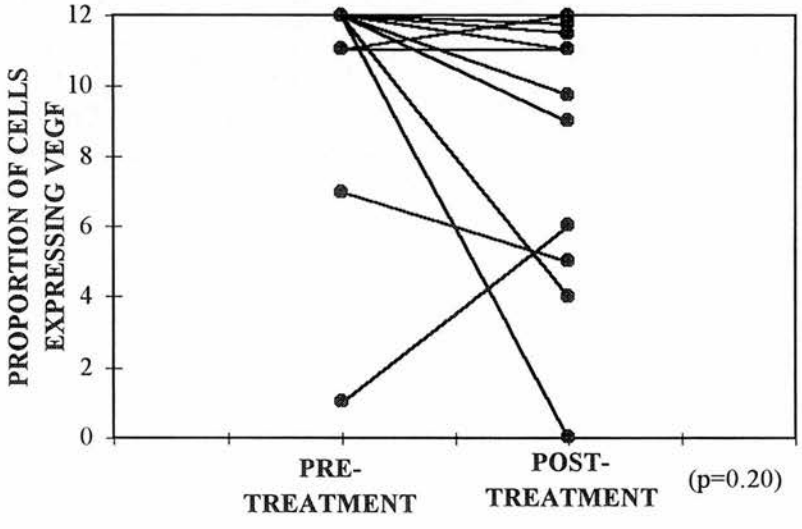


Table 5.1.4: Comparison of proportion of cells expressing VEGF before and after treatment in non-responding tumours.

	Pre-treatment	Post-treatment
Range	1-12	0-12
Median	12	10
Mean	10.5	8.5
Standard error	0.96	1.13
Standard deviation	3.32	3.90

Mann-Whitney test: p=0.2

Changes in % of VEGF-postive tumour cells with response to tamoxifen.

The numbers of tumours in which the proportion of tumour cells staining for VEGF increased, remained static or decreased are tabulated below. No significant trend was observed in association with response. In both non-responders and responders the proportion of positively staining cells tended to reduce with treatment.

Table 5.1.5: Changes in VEGF expression in non-responding and responding tumours following treatment with tamoxifen:

	Increased VEGF	VEGF static	Decreased VEGF
Non-responders	2	3	7
Responders	11	6	19

5.2 % VEGF-positive tumour cells and microvessel counts.

The relationships between %VEGF-positive cells and microvessel counts before and after treatment with tamoxifen were investigated and are illustrated on Figures 5.2.1 and 5.2.2. No significant correlation was found. In addition, no significant correlation was found between the changes in mvc and VEGF expression in each tumour (r=-0.03), as tabulated on Table 5.2.1.

Figure 5.2.1 : Relationship between % VEGF-positive cells and microvessel count before treatment.

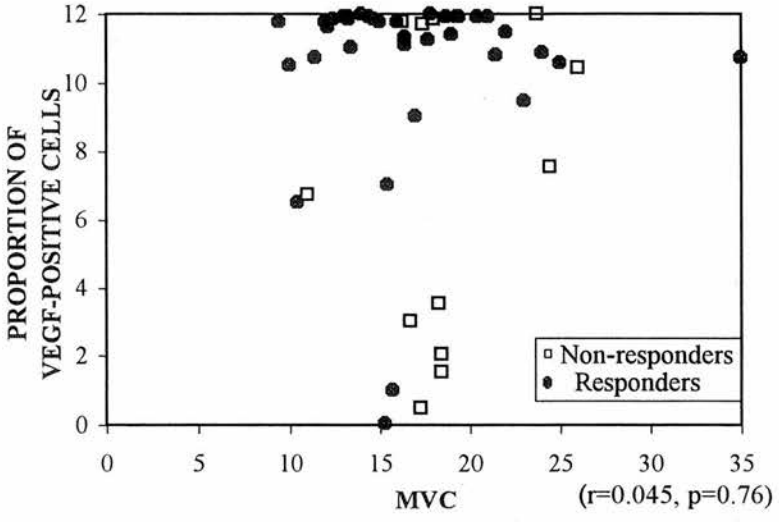


Figure 5.2.2 : Relationship between % VEGF-positive cells and microvessel count after treatment.

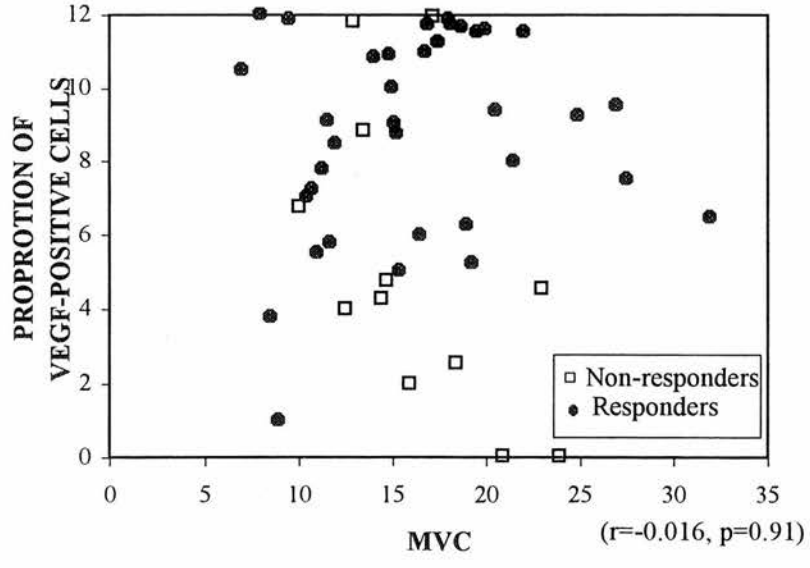
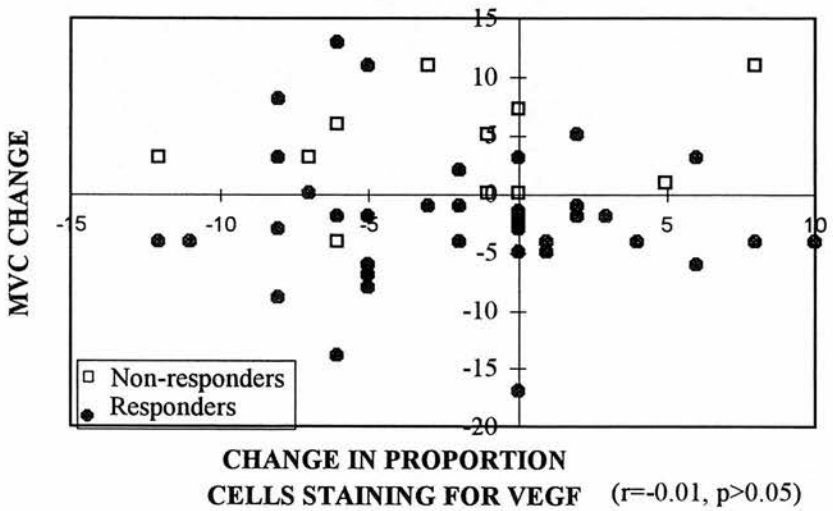


Table 5.2.1: Comparison of changes in mvc and VEGF expression following treatment with tamoxifen: where 0=no change, 1=decrease, 2=increase. Non-responders in **bold**.

CHANGE IN MVC	CHANGE IN VEGF EXPRESSION
1	**0**
2	**0**
0	**0**
2	**1**
2	**1**
1	**1**
2	**1**
2	**1**
2	**1**
0	**1**
0	**2**
2	**2**
1	0
1	0
1	0
1	0
1	0
2	0
1	1
1	1
1	1
1	1
2	1
1	1
2	1
1	1
2	1
0	1
1	1
0	1
1	1
2	1
1	1
1	1
1	1
2	1
0	1
1	2
1	2
1	2
2	2
1	2
0	2
1	2
1	2
2	2
1	2
1	2

In addition, there was no correlation between degree of change in mvc and % VEGF-positive cells, as illustrated in Figure 5.2.3 (r=-0.01).

Figure 5.2.3: Relationship between change in % tumour cells staining for VEGF and difference in mvc following treatment.



5.3. Discussion:

Work described in this thesis has focused on changes in breast tumour vascularity with tamoxifen by the assessment of microvessel densities in tumour specimens. An alternative method of assessing changes in tumour vascularity is to study growth factors responsible for stimulating tumour angiogenesis. Many such growth factors exist: in the majority, their effect on endothelial cells represents only a part of their total action. Vascular endothelial growth factor (VEGF) is unusual in that it acts solely on endothelial cells (Ferrara & Henzel, 1989). Produced by normal cells, its expression is markedly increased in breast cancers (Relf *et al*, 1997).

In this study, changes in the number of cells staining for VEGF that occurred following treatment of breast cancers with tamoxifen for between three and six months were investigated and correlated with response. In addition, the relationship between number of VEGF-positive cells and microvessel counts performed on the same tumours was studied. Forty-eight patients were studied: 12 (25%) had non-responding and 36 (75%) had responding tumours. Prior to treatment, the majority of tumour cells expressed VEGF and there was no difference in % positive cells between non-responding and responding tumours. Changes in the number of VEGF-positive cells were observed following treatment with tamoxifen. In both non-responding and responding tumours, there was a trend for a reduction in the number of cells staining for VEGF following treatment, but this was more marked in responding than in non-responding tumours. There was a significant reduction in number of cells staining in responding tumours ($p=0.03$) following treatment, but the change in non-responding tumours failed to reach statistical significance.

There was no significant correlation between microvessel counts and % VEGF-positive tumour cells before or after treatment. In addition no correlation was found between the degree of change in number of cells expressing VEGF and the difference in mvc following treatment.

In colorectal carcinomas, VEGF expression correlated with the extent of neovascularization and proliferation, whereas bFGF did not (Takahashi *et al*, 1995). In a study of small cell lung cancer microvessel counts were strongly associated with VEGF and altered p53 expression, suggesting that wild type p53 regulates angiogenesis through VEGF (Macchiarini *et al*, 1992). Indeed mutations of p53 result in increased VEGF expression (Keiser *et al*, 1994).

Several studies have found an association between VEGF expression and relapse-free or overall survival in breast cancers, with significantly shorter survival times in patients with VEGF-rich tumours (Gasparini *et al*, 1997; Toi *et al*, 1995). Studies investigating effects of tamoxifen on VEGF expression have been limited to experimental systems, but have demonstrated inhibition of VEGF-stimulated endothelial cell growth (Gagliardi *et al*, 1996; McNamara *et al*, 1998). The reduction in number of cells expressing VEGF in treated breast cancers observed in the present study is in keeping with these findings.

A clear pattern of change in number of cells staining for VEGF was observed in the present study, with a reduction in % cells staining in the majority of tumours, regardless of response. The more marked reduction of VEGF-expressing tumour cells in responding than non-responding tumours suggests that a percentage of tumour cells respond to tamoxifen by "switching off" VEGF expression but some cells fail to do so. It may be that cells continuing to express VEGF after treatment represent clones that are resistant to tamoxifen.

The failure of the present study to demonstrate a positive correlation between VEGF expression and microvessel density is in contrast to most other published series (Anan *et al*, 1996; Toi *et al*, 1995). This may be related to the methods adopted to assess VEGF, which vary markedly, including frequency of mRNA expression, in which VEGF was either present or absent (Anan *et al*, 1996) and enzymatic immunoassay of tumour cytosol (Gasparini *et al*, 1997; Toi *et al*, 1996). In studies adopting immunohistochemical techniques for assessment of VEGF, intensity of staining alone has been most commonly used (Toi *et al*, 1995). Not all studies have found a correlation between VEGF expression and mvc: de Jong et al investigated a range of growth factors in relation to proliferation and angiogenesis and VEGF expression failed to correlate with either parameter (de Jong *et al*, 1998).

Changes in number of positively staining cells for VEGF in this group of patients were less significant than changes in microvessel count, as detailed earlier in the thesis. VEGF is one of several growth factors known to stimulate angiogenesis. The process of angiogenesis represents a balance in activity of inhibitors and inducers, which is affected by processes other than tumour growth. Angiogenesis is stimulated by hypoxia, mediated by VEGF, and forms part of the physiological processes of wound healing. Changes in VEGF thus form a small part of the processes occurring within the responding tumour, which may provide an explanation for it being less significant than mvc in determining response. In addition, it

must be noted that the number of tumours, particularly non-responding tumours, is small for statistical analysis.

In summary, the proportion of tumour cells staining for VEGF was reduced following treatment of primary breast cancers with tamoxifen in responding tumours. There was also a trend for reduction in % cells staining in non-responding tumours. Cells which were rendered VEGF-negative following treatment with tamoxifen may represent those that were sensitive to treatment. Failure of correlation of %VEGF-positive cells and mvc is contrary to the findings of most published series but these have varied in methodology and in definition of VEGF expression.

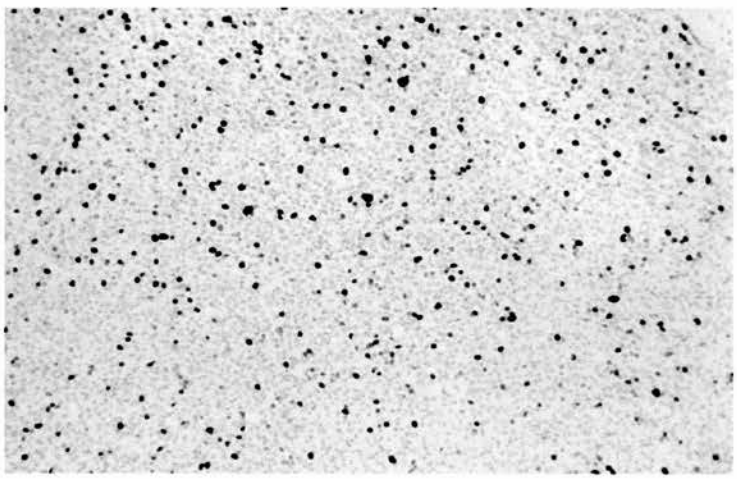
6. Changes in tumour cell proliferation during tamoxifen treatment.

The main focus of this thesis has been on changes in tumour vascularity in association with primary tamoxifen treatment. Results described previously have demonstrated that assessments of tumour vascularity failed to provide a useful early marker of response to tamoxifen. Reasons for this are thought to be due to problems of reproducibility of counts in different breast cancer specimens, but it may be that changes do not occur in tumour biology early in the course of treatment. A study has been undertaken to determine whether an alternative marker of response assessed on small tumour biopsies may provide useful early information regarding response to tamoxifen.

The % of tumour cells undergoing proliferation was assessed following staining with MIB-1 antibody before, during and after treatment with tamoxifen. These assessments were made on tumours that were previously studied in Results chapter 4 of the thesis. Forty-one patients were studied: one patient was excluded due to technical problems with the staining. Thirteen patients had tumours that failed to respond to tamoxifen and twenty-eight had responding tumours, yielding an overall response rate of 67%.

Following immunohistochemical stainign with MIB-1 antibody to Ki67, assessments were made of % positively staining tumour cells staining in the entire tumour section. An example of MIB-1 staining is illustrated in Figure 6.1. Tumours were scored in categories up to a maximum score of 5. A score of 0 was given if no cells stained positively, 1 if <1% cells were positive, 2 if 1-10% cells were positive, 3 if 11-33% cells were positive, 4 if 34-66% cells were positive and 5 if >67% cells were positive for MIB-1 antibody. Scores were compared before, during and after completion of treatment with tamoxifen and correlated with response.

Fig 6.1. Example of staining with proliferation marker Mib-1 antibody (low power x 40)



6.1. Proliferation scores and response.

Pre-treatment MIB-1 scores: The proliferation scores in non-responding and responding tumours prior to treatment are compared in Figure 6.1.1, demonstrating no significant difference between the two groups (p=0.25). The range of score in non-responding tumours was 2-4 (median: 3, mean: 2.9) and in responding tumours was 2-5 (median: 2, mean: 2.6). Data are summarised on Table 6.1.1 and detailed data are shown in the appendix to this chapter.

Figure 6.1.1: Comparison of MIB-1 expression in non-responding and responding tumours before treatment.

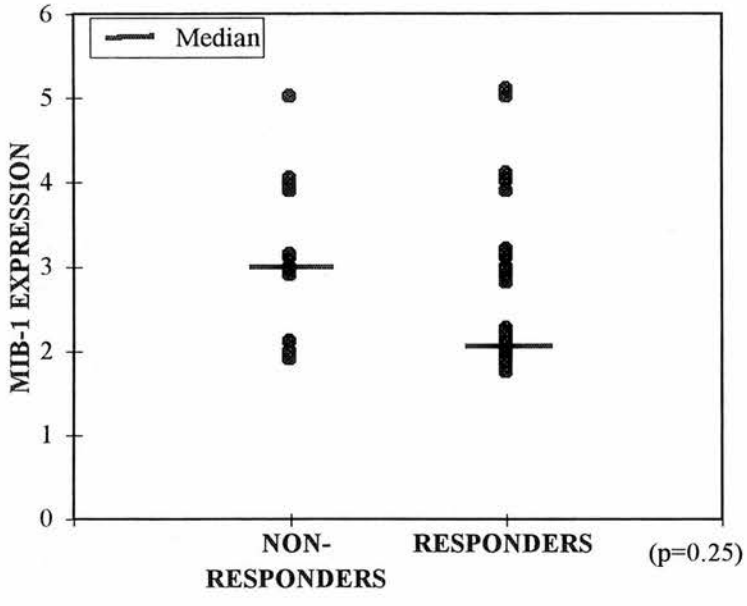


Table 6.1.1: Comparison of pre-treatment MIB-1 scores in non-responding and responding tumours.

	Non-responders	Responders
Range	2-4	2-5
Median	3	2
Mean	2.9	2.6
Standard error	0.21	0.16
Standard deviation	0.76	0.83

Mann-Whitney test: p=0.25

Peri-treatment MIB-1 scores: MIB-1 scores of tumour sections taken following two weeks' treatment with tamoxifen are compared in non-responding and responding tumours in Figure 6.1.2. The range of scores in non-responding tumours was1-4 (median: 3, mean: 2.67) and in responding tumours was 1-5 (median: 2, mean: 2.16). There was no significant difference between the two groups (p=0.11). Data are outlined in Table 6.1.2.

Figure 6.1.2: Comparison of MIB-1 expression in non-responding and responding tumours during treatment.

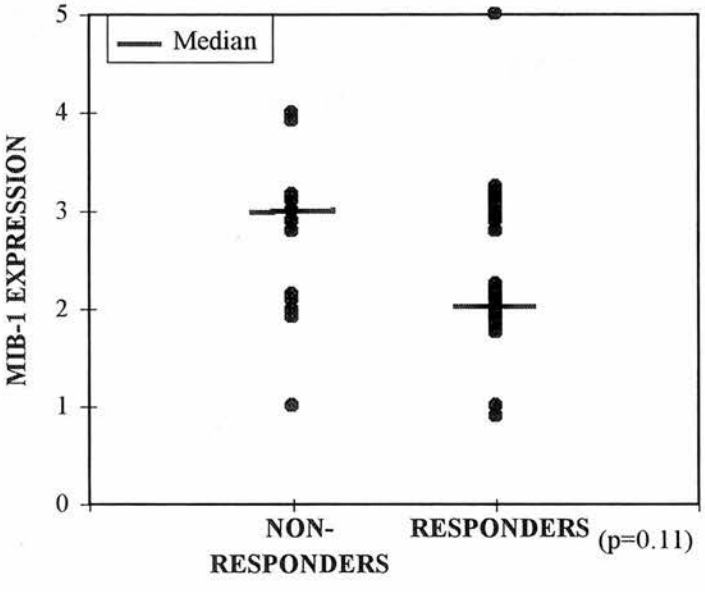


Table 6.1.2: Comparison of peri-treatment MIB-1 scores in non-responding and responding tumours.

	Non-responders	Responders
Range	1-4	1-5
Median	3	2
Mean	2.67	2.16
Standard error	0.26	0.19
Standard deviation	0.89	0.94

Mann-Whitney test: p=0.11

Post-treatment MIB-1 scores: MIB-1 scores were compared in non-responding and responding tumours on completion of three months' treatment with tamoxifen, and demonstrated a significant difference between the two (p=0.023). The range of MIB-1 scores in non-responding tumours was 2-5 (median: 3, mean: 3.17) and in responding tumours was 1-4 (median: 2, mean: 2.11). Data are outlined on Table 6.1.3.

Figure 6.1.3: Comparison of MIB-1 expression in non-responding and responding tumours following treatment.

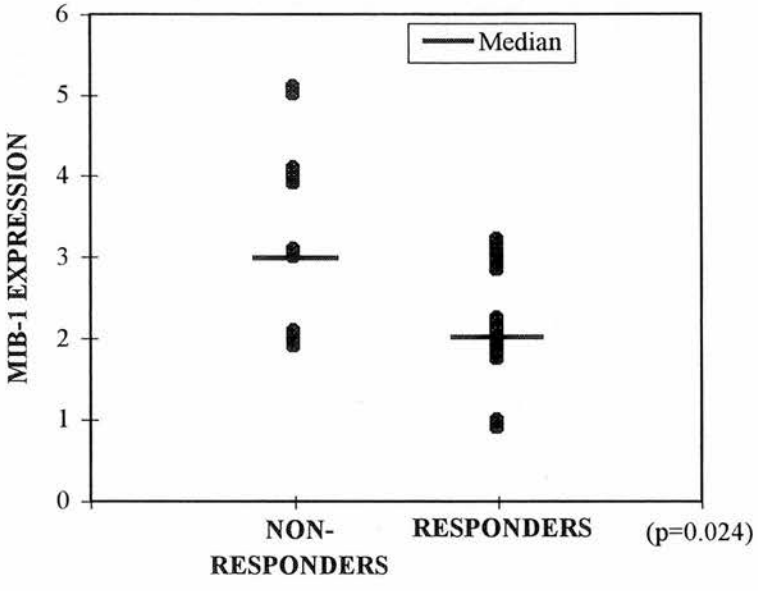


Table 6.1.3: Comparison of post-treatment MIB-1 scores in non-responding and responding tumours.

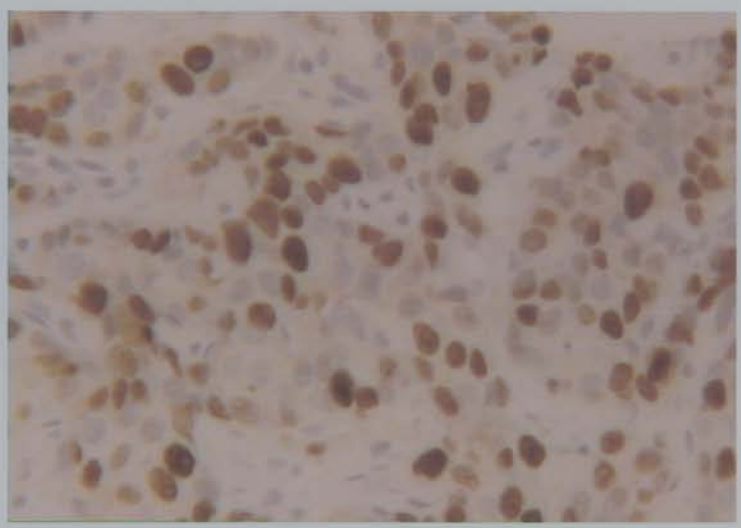
	Non-responders	Responders
Range	2-5	1-4
Median	3	2
Mean	3.17	2.11
Standard error	0.34	0.17
Standard deviation	1.19	0.89

Mann-Whitney test: p=0.024

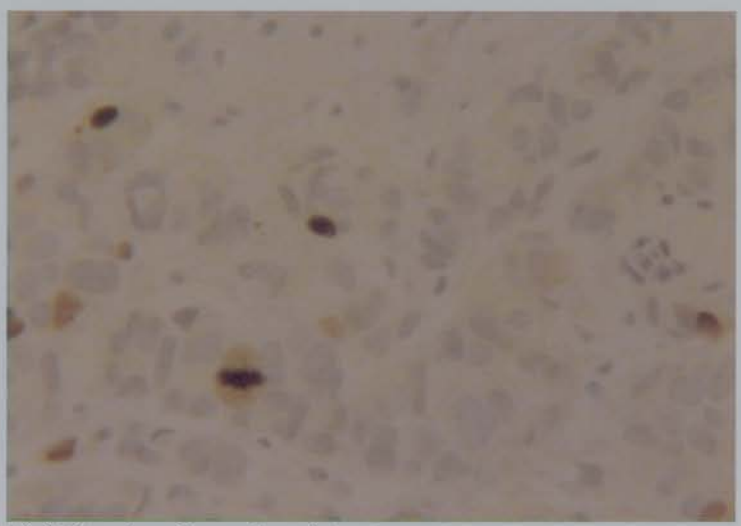
Changes in MIB-1 expression during treatment with tamoxifen.

Changes in % proliferating cells occurred in non-responding and responding tumours, as demonstrated in Figure 6.1.4.

Fig 6.1.4 Reduction in proliferation following two weeks treatment with Tamoxifen. (high power x100)



a) before treatment



b) following 2 weeks of treatment

Non-responding tumours: MIB-1 scores were compared in non-responding tumours before, during and after treatment with tamoxifen. There was no significant trend in change of MIB-1 score throughout treatment, as illustrated on Figure 6.1.5. Data are outlined on Table 6.1.4.

Figure 6.1.5: Changes in MIB-1 expression in non-responding tumours during treatment with tamoxifen.

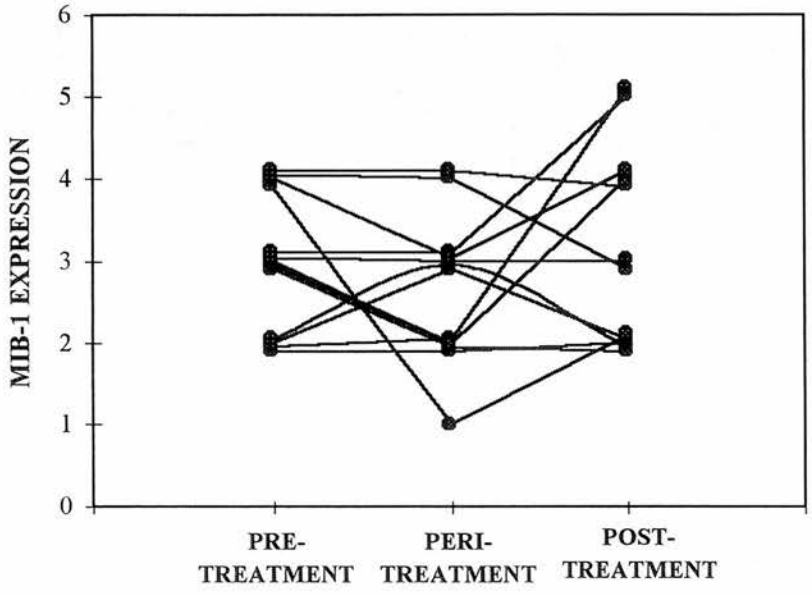


Table 6.1.4: Changes in MIB-1 expression in non-responding tumours:

	Pre-treatment	Peri-treatment	Post-treatment
Range	2-4	1-4	2-5
Median	3	3	3
Mean	2.9	2.67	3.17
Standard error	0.21	0.26	0.34
Standard deviation	0.76	0.89	1.19

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment MIB-1 expression: p=0.44

Pre- v. post-treatment MIB-1 expression: p=0.58

Peri- v. post-treatment MIB-1 expression: p=0.47

Responding tumours: There was a significant reduction in MIB-1 expression following two weeks' treatment with tamoxifen (p=0.015). This reduction persisted throughout treatment and remained significant at three months (p=0.006). There was no significant difference in count in tumour taken following two weeks of treatment and tumour taken on completion of three months' treatment (p=0.85). Changes in MIB-1 expression are illustrated on Figure 6.1.6, with data outlined on Table 6.1.5.

Figure 6.1.6: Changes in MIB-1 expression in responding tumours during treatment with tamoxifen.

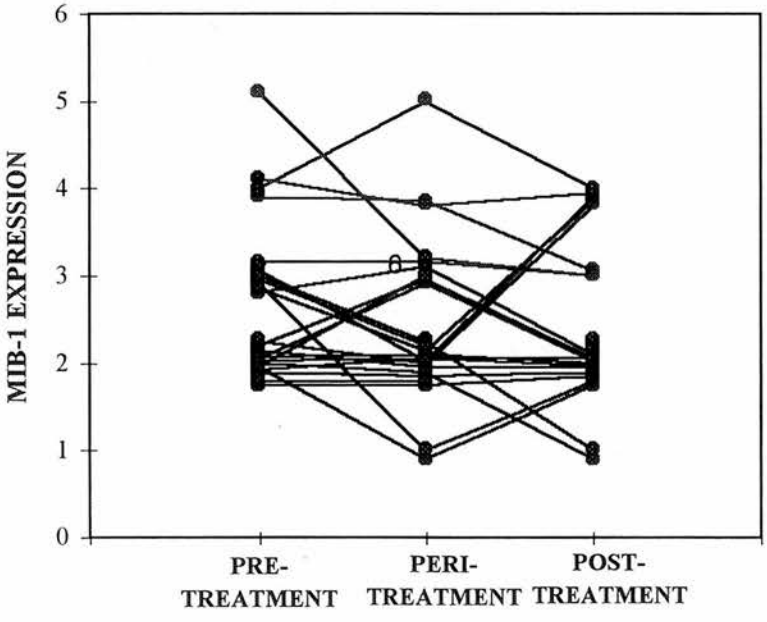


Table 6.1.5: Changes in MIB-1 expression in responding tumours:

	Pre-treatment	Peri-treatment	Post-treatment
Range	2-5	0-5	0-4
Median	2	2	2
Mean	2.6	2.16	2.11
Standard error	0.16	0.19	0.17
Standard deviation	0.83	0.94	0.89

Paired Wilcoxon signed rank test:

Pre- v. peri-treatment MIB-1 expression: p=0.02

Pre- v. post-treatment MIB-1 expression: p=0.006

Peri- v. post-treatment MIB-1 expression: p=0.85

Changes in MIB-1 expression with response to tamoxifen.

The number of patients in whom MIB-1 expression increased, remained static or decreased between the different time points is outlined in Tables 6.1.6 and 6.1.7. Whilst there was no significant trend observed at the early time point in responding tumours, there was a significant trend for change in MIB-1 expression at three months (p=0.047).

Table 6.1.6: Early changes in MIB-1 expression in non-responding and responding tumours (Pre v. peri-treatment scores):

	Increased MIB-1	**MIB-1 static**	**Decreased MIB-1**
Non-responders	2	6	4
Responders	5	9	11

Chi squared for trend: p=0.78

Table 6.1.7: Changes in MIB-1 expression in non-responding and responding tumours at three months (Pre v. post-treatment scores):

	Increased MIB-1	**MIB-1 static**	**Decreased MIB-1**
Non-responders	4	5	3
Responders	1	15	11

Chi squared for trend: p=0.047

6.2 Discussion.

In the prospective study described earlier in the thesis, changes in microvessel count in sequential tumour biopsies failed to provide early predictive information on response to tamoxifen. Indeed results of the study failed to reproduce findings of the previous retrospective study in which changes in mvc occurred in association with response to tamoxifen. This is likely to be related to methodological problems due to the small size of tumour biopsies taken.

In the present study an alternative potential marker of early response was investigated which is not dependent on selection of 'hot spots' for the assessment and is less susceptible to tumour heterogeneity. Changes in percentage of proliferating cells following staining with MIB-1 antibody were determined and correlated with response in the same cohort of patients as detailed previously.

There was no difference in MIB-1 scores between non-responding and responding tumours at any of the three time points. There was, however, a significant reduction in score in responding tumours following two weeks of treatment, a change that became more significant following three months' treatment with tamoxifen. The difference in score in responding tumours between the biopsy at two weeks and that taken at three months was not significant. No significant change in proliferation in non-responding tumours was observed at any of the time points. There was no significant trend in change in responding tumours at the early time point but the trend reached statistical significance following three months of treatment.

The anti-proliferative action of tamoxifen has been well-documented. It was first demonstrated in vitro for the hormone-sensitive human breast cancer derived cell line, MCF-7: an effect that could be reversed by addition of oestrogen (Lippman & Bolan, 1975). Studies have demonstrated a reduction in proliferation in xenograft (Cameron *et al*, 1997b) and clinical tumours (Keen *et al*, 1997) following treatment with tamoxifen. In the study of clinical tumours, there was no correlation between the reduction in proliferation and percentage reduction in tumour volume. Proliferation was assessed following staining with KiS1 antibody, which may vary with different concentrations of antibody (Keshgegian & Cnaan, 1995). Ki-67 (MIB-1) is more robust and is not expressed in non-proliferating cells. A recent study has adopted use of sequential fine needle aspirations to obtain samples prior

to commencement, at 14 days and at eight weeks' post-treatment with tamoxifen (Makris *et al*, 1998). There was a significant difference in Ki-67 expression in responding versus non-responding tumours at the early time point that was not maintained throughout the study. This evidence from previous studies suggested that changes in proliferation during treatment with tamoxifen might indeed provide a useful early marker of response. The present study is the first to adopt the methodology of performing sequential core biopsies during treatment.

Findings in the present study are in keeping with previously published work, with a reduction in percentage of proliferating cells in responding tumours. In the present study such a reduction was maintained throughout the study, with increasing significance. There were, however, some patients with non-responding tumours in whom MIB-1 expression fell, particularly in the first two weeks of treatment. This is in keeping with the findings of the study of FNA samples in which there was a slight fall in Ki-67 expression in non-responding tumours following 14 days of treatment (Makris *et al*, 1998). Thus it may be that the degree of reduction in expression is important. The current method of categorising scores may not be sufficiently sensitive to detect such differences; it may be that a continuous method would provide more information. The current method of assessment is under consideration.

These findings are of interest and suggest that changes do take place within the tumour early during treatment with tamoxifen in association with subsequent response, although such changes were not demonstrated in tumour vascularity. The potential role of changes in MIB-1 expression as an early marker of response warrants further investigation. In particular it will be important to assess the reproducibility of the method in a similar manner to the study of reproducibility of microvessel counting as described in this thesis. Such work is ongoing.

Discussion

This study focused on changes in tumour vascularity that are consequent upon tamoxifen treatment of breast cancer and aimed to determine whether early changes in tumour vascularity during neoadjuvant tamoxifen treatment provided a useful predictor of response. Before embarking upon studies investigating the effect of tamoxifen on angiogenesis, a series of control experiments was undertaken. The aims of these were to validate the methodology of microvessel counting, to assess reproducibility of counts in different breast cancer specimens and to compare the use of two antibodies to endothelial cells, antibody to Factor VIII and to CD31.

Results of the studies aimed at validating the methodology demonstrated low observer variation, in keeping with other studies (Martin *et al*, 1997), and a highly significant correlation in counts performed following staining with antibody to Factor VIII and CD31, which has also been previously demonstrated (Horak *et al*, 1992). Comparison of counts in core biopsy specimens versus tumour cross-sections has not been previously described in detail. A positive correlation was found between the two counts when they were performed simultaneously, but there was no correlation when they were performed two weeks apart with no intervening treatment. This lack of reproducibility of counts in different specimens is likely to be related to sampling problems due to tumour heterogeneity and to the small size of core biopsy specimens. It may be that the accepted method of performing microvessel counts in areas of high vascularity is not appropriate in small tumour specimens, particularly in the present study, in which the focus has not been to assess the prognostic value of microvessel counts.

A series of experiments was undertaken to assess the effect of tamoxifen on tumour vascularity. In order to overcome problems of methodology, tamoxifen would have to exert a dramatic effect on vascularity. A comparison of microvessel counts before and after treatment in a retrospective study of fifty-seven patients demonstrated a significant reduction in mvc in responding tumours and a significant increase in non-responding tumours following treatment. These changes were observed when clinical behaviour of the tumours was already known and may be the result of response or failure of response rather than the precedent of it. In order to be of value as a predictor of response to therapy, changes at an early time point would be required.

To assess the timing of such changes in tumour vascularity relative to tumour regression a series of xenograft studies was undertaken. The study of ER-positive and ER-negative breast cancer xenografts treated with tamoxifen revealed a reduction in microvessel counts in ZR-75 ER-positive tumours but not in MDA-MB-231 ER-negative tumours. The reduction occurred following two days' treatment with tamoxifen and preceded tumour regression. These findings supported both the results of the early clinical study and the hypothesis that early changes in tumour vascularity may predict for response to endocrine therapy.

It was against this background of reduced mvc in responding tumours on completion of primary tamoxifen and evidence from an animal model that such a reduction occurred as an early event during the course of treatment that a prospective study was undertaken. The aim of the study was to determine whether, in the clinical setting, early changes predicted for response, despite limitations in the methodology detailed previously. Sequential core biopsies were performed during a three month course of primary treatment with tamoxifen, and microvessel counts carried out at the three time points. The study demonstrated increased mvc in non-responding tumours following two weeks' treatment when compared with pre-treatment counts, following staining with antibody to Factor VIII but not CD31. No significant trend in responding tumours was observed at the early time point. In addition, the study failed to reproduce findings of the earlier retrospective study in which there was a significantly reduced mvc in responding tumours and an increase in non-responding tumours at the end of three months' treatment.

The increase in mvc in non-responding tumours found on completion of three months' treatment in the retrospective study and seen as an early change following staining with antibody to Factor VIII in the prospective study is worthy of note. It may be a reflection of growth of the unresponsive tumour, but there is a possible concern that tamoxifen may be stimulating tumour growth in this subgroup of patients. It is possible that such stimulation occurred as a result of tamoxifen exerting oestrogenic action in this subgroup of patients.

The reasons for the failure of the prospective study to either identify early changes in tumour vascularity in association with response or to reproduce findings of the earlier study are likely to be related to the methodology. Core biopsies are easy to perform in the out-patient setting and can be repeated, but provide less volume of tumour than the wedge biopsies which were performed in the retrospective study. Tamoxifen does not exert a sufficiently large effect on tumour vascularity to overcome problems with reproducibility that were

identified in the studies addressing this issue detailed earlier in this thesis. The problems of heterogeneity of tumour vascularity and the likely effect of previous surgical manipulation in stimulating angiogenesis as part of wound healing, mask any effect of tamoxifen in these biopsy specimens. It may be more appropriate to perform random counts in these specimens rather than adhering to the recommendations made by previous authors that counts should be performed in vascular 'hot spots', particularly as this study did not attempt to address the prognostic value of microvessel counting.

In studies addressing the issue of prognosis, not all have found microvessel counts to correlate with prognosis or with other prognostic indicators (Costello *et al*, 1995). A possible explanation for this is that, until recently, microvessel counts have been performed following staining with a pan-endothelial cell marker. A recent study has suggested that the ability to distinguish between tumour neovascularization and pre-existing new vessels may be important and demonstrated the potential role of an antibody (CD105) that specifically stains angiogenic endothelial cells (Kumar *et al*, 1999). This new antibody requires further study and whilst such controversy remains it may that an alternative method of assessment of tumour vascularity may provide an early marker of response to primary therapy: for example, the expression of angiogenic factors.

Tumour expression of the angiogenic inducer, vascular endothelial growth factor (VEGF), is likely to be less susceptible to the effect of tumour heterogeneity than the vessels themselves as assessment does not require selection of vascular 'hot spots'. VEGF is expressed by tumour cells and acts as a specific endothelial cell mitogen. Thus, having identified that the assessment of tumour vascularity by microvessel counting is unlikely to provide useful information as a predictor of response to endocrine therapy, the % of VEGF-positive tumour cells was investigated in the same tumours. There was a significant reduction in % cells staining in responding tumours following three months' treatment with tamoxifen, but no significant change was observed in non-responding tumours. In the majority of tumours studied, VEGF was expressed in nearly all tumour cells before treatment, but there was a reduction in percentage cells staining after treatment in both non-responding and responding tumours. This suggests that a percentage of tumour cells were sensitive to tamoxifen in both groups and one aspect of their response was to "switch off" VEGF expression. It may be that the cells that persisted in VEGF expression may represent resistant clones, which were more numerous in non-responding than responding tumours. However, the number of non-responding tumours studied was small.

Despite the lack of clear difference in the effect of tamoxifen on % tumour cells staining for VEGF in non-responding and responding tumour in this study it would be of value to investigate its reproducibility in breast cancer specimens, which may be less affected by tumour heterogeneity. The value of early changes in VEGF expression as an early predictor for response could then be investigated. Such work is ongoing.

Work in this thesis has not attempted to ascertain the mode of action of tamoxifen on tumour angiogenesis. Other investigators have addressed this issue in experimental systems and have demonstrated a direct anti-angiogenic effect that was not mediated by the oestrogen receptor (Gagliardi *et al*, 1995). Tamoxifen inhibited endothelial cell growth stimulated by angiogenic growth factors such as VEGF and bFGF (Gagliardi *et al*, 1996; McNamara *et al*, 1998). Results of the xenograft studies detailed in this thesis suggest that effects of tamoxifen were related to ER expression because no reduction in mvc was observed in ER-negative MDA-MB-231 tumours. In the clinical studies letrozole appeared to exert a similar effect on tumour angiogenesis, suggesting that the effect was not specific to tamoxifen, but may be a reflection of oestrogen deprivation.

The main focus of work in this thesis has been based on a single method of assessing tumour vascularity: microvessel counting. Changes in % tumour cells expressing VEGF following treatment have also been studied, but were found to be less significant than mvc in the retrospective study. Further investigation of VEGF expression in the prospective study would be of value, however, particularly as the effects of tumour heterogeneity are likely to be less marked. An alternative method of assessing angiogenic factor expression would be by serial biochemical measurements of growth factors in the serum or urine of patients during treatment. Elevated levels of VEGF and bFGF have been found in sera of patients with metastatic colorectal, ovarian and renal carcinomas (Dirix *et al*, 1997). Another non-invasive method of angiogenesis assessment includes colour Doppler ultrasound that was studied in too small a number of patients in this thesis for meaningful comment. An alternative method is contrast-enhanced magnetic resonance imaging (MRI), which is currently under investigation. MR enhancement has been found to be associated with high vessel densities, but data showed considerable variation and was therefore not considered to be an accurate predictor of microvessel density (Buckley *et al*, 1997; Stomper *et al*, 1997). In another study of MRI in combination with a contrast agent directly targeting the endothelial integrin $\alpha V\beta 3$ provided enhanced and detailed imaging of rabbit carcinomas, including

identification of angiogenic hot spots, which had not been previously detected by MRI (Sipkins *et al*, 1998). The development of new contrast agents and technological improvements in MRI may provide an important non-invasive method of assessing tumour angiogenesis.

The strength of the model adopted in the present study is that tumour biopsy samples were available at the early time point of 14 days after commencement of endocrine treatment in addition to those available before and after completion of primary endocrine therapy. Few patients invited to participate in the study declined a second biopsy, despite explanation of the research role of such biopsies. The use of local anaesthetic before performing the biopsies is thought to be an important factor for the patient. Local anaesthetic infiltration is not performed prior to FNAs.

This study has provided a valuable source of material for investigation of other potential early markers of sensitivity. Before embarking on further studies, however, it is important to determine whether the failure of microvessel counts to provide an early marker of response was related to the methodological issues described within this work, or whether the time point of two weeks was too early to detect any changes in tumour biology. It is for this reason that changes in tumour cell proliferation during treatment were studied. In this study, there was a significant reduction in MIB-1 expression in responding tumours at two weeks, but not in non-responding tumours. This reduction persisted throughout the course of treatment, in contrast to a previous study in which early reduction in proliferation assessed on FNA samples did not persist at eight weeks (Makris *et al*, 1998). This finding of reduced MIB-1 expression early in the course of treatment is encouraging and confirms that early changes in tumour biology do take place which can be detected on core biopsy specimens. Studies assessing the reproducibility if MIB-1 assessments are ongoing. In addition, other potential early markers of response are being investigated in the same group of patients. Current work involves assessment of apoptosis, which will enable calculation of the apoptotic: mitotic ratio, which has been shown to be important in predicting response to endocrine therapy in xenograft studies (Cameron *et al*, 1997b). It is hoped that assessment of a range of potential markers of sensitivity will lead to the establishment of a predictive index of sensitivity, which will enable clinicians to stop ineffective treatment at an earlier time point than is currently possible.

In conclusion, the studies described in this thesis have demonstrated a change in vascularity of primary breast cancers following three months' treatment with tamoxifen. Xenograft studies showed these changes occurred early during treatment with tamoxifen in ER-positive ZR-75 tumours, but not ER-negative MDA-MB-231 tumours, suggesting that such changes may be of benefit in the clinical setting as an early predictor of response to endocrine therapy. However, a prospective study adopting the use of sequential core biopsies before, during and after primary treatment with tamoxifen failed to demonstrate early changes in tumour vascularity which would predict for response. Results of a series of control experiments suggested that lack of reproducibility of mvc in different breast cancer specimens might be the cause of this failure. However, tumour biopsies collected during the study provide valuable material for evaluation of other potential markers. Other methods of assessing angiogenesis are available, as well as markers of proliferation and apoptosis. It is hoped that ongoing work adopting the model outlined in this thesis may yield a clinically useful index for predicting sensitivity to primary endocrine therapy.

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Appendices

Appendix to Materials and Methods.

Materials:

Animal experiments:

HsdOla: ICRF-nu mice	HARLAN (UK)
Standard diet	HARLAN (UK)
Slow-release oestrogen pellet (0.72mg, released over 60 days)	INNOVATIVE RESEARCH OF AMERICA
Slow-release tamoxifen (2.5mg, released over 60 days)	INNOVATIVE RESEARCH OF AMERICA

Preparation of all specimens:

Neutral-buffered formalin	FISHER F/1510/21
Superfrost microscope slides.	BDH 406/0179/00
Glass coverslips(22x40mm)	CHANCE PROPPER LTD

Reagents for dewaxing of sections:

Xylene:	FISHER X/0100/17	
Absolute alcohol:	FISHER E/0600/17	Lot number: 9751849 307

Staining for histological assessment:

Haematoxylin solution	SIGMA HHS-32
Eosin solution	SIGMA HT 110-1-32

Reagents for immunohistochemstry:

Tris buffered saline (TBS):

TRIZMA base.	SIGMA T-1503	Lot number: 16H5738
Sodium chloride.	FISHER S/3120/53	Lot number: 9741598 227

0.9% solution prepared in distilled water, by adding 9g sodium chloride to 1 litre distilled water.

Hydrochloric acid FISHER H/1000/PB17

To make up TBS: 60.55g Trizma base was added to 1 litre distilled water and pH optimized to

7.6 by addition of concentrated hydrochloric acid.

0.05M TBS was used for staining; to achieve this concentration, 100mls TBS was added to 900ml 0.9% sodium chloride.

Phosphate buffered saline.

Dulbecco 'A' tablets: DULBECCO B1R14a Lot number: R035614-002.
OVOID Unipath Ltd.

A single tablet was dissolved in 100mls distilled water to make up PBS solution.

Fetal calf serum. GIBCO

Hydrogen peroxide. SIGMA H-1009 Lot number: 16H0355

Reagents for antigen retrieval:

Protease Type XXIV SIGMA P-8038 Lot number: 86H0561

Citric acid SIGMA C-7129 Lot number: 96H0355

Trypsin powder DIFCO 0152-13-1 Lot number: 50077JC

Trypsin tissue culture (x10) SIGMA T-4549 Lot number: 17H2373

Dilution: 10mls trypsin in 90mls TBS.

Primary antibodies:

Mouse anti-human CD31 monoclonal antibody. Clone JC/70A
DAKO M0823 Lot number: 016 (201)

Anti-Von Willebrand factor monoclonal antibody. Clone F8/86.
DAKO M0616 Lot number: 126 (101)

Rat anti-mouse CD31 monoclonal antibody (MEC13.3).
PHARMINGEN 01951D Lot number: M022293

Polyclonal rabbit anti-VEGF antibody
SANTA CRUZ BIOTECHNOLOGY Code sc: 507

MIB-1 antibody BIOGENEX

Secondary antibodies:

Rabbit anti-mouse immunoglobulin: DAKO Z0259 Lot number: 067 (101)

Rabbit anti-rat immunoglobulin (APAAP): DAKO Z0494 Lot number: 072 (401)

Rabbit anti-rat immunoglobulin (biotin): DAKO E0468 Lot number: 045 (301)

StrepABComplex/HRP duet mouse/rabbit kit containing biotinylated goat anti-mouse IgG
DAKO Code K0492

Vector anti-mouse.

Tertiary systems:

APAAP, mouse, monoclonal:	DAKO D0651	Lot number: 106 (101).
APAAP, rat, monoclonal:	DAKO D0488	Lot number: 046 (102).

Streptavin Peroxidase (HRP conc) BIOGENEX LP000-UL

Visualization systems:

New Fuchsin Substrate system:	DAKO K698
Diaminobenzidine: (DAB)	Kem-En-Tec 4240

Mounting media:

Aquamount	BDH 362262H	Lot number: 70213773.
DPX mounting medium:	FISHER D/5319/05	Lot number: 9624511 386.

APAAP STAINING PROTOCOL USING JC 70 ANTI-CD31 MOUSE ANTIBODY.

1. Freshly excised breast tissue fixed in 10% formal saline, processed and embedded in paraffin wax.
2. 5μm paraffin sections cut in routine histology laboratory.
3. Dewax in xylene (xylene 1: 5 minutes, xylene 2: 5 minutes) and rehydrate through graded alcohols (100% ethanol: 5 minutes, 99% ethanol: 5 minutes, 95% ethanol: 5 minutes, distilled water: 10 minutes).
4. Pre-incubate sections in distilled water warmed to 37°C.
5. Make up 200ml PBS (pH7.4) and pre-warm to 37°C in incubator.
6. Dissolve 25mg protease type XXIV in 200ml warm PBS and incubate slides in protease solution for 20 minutes at 37°C.
7. Transfer slides to distilled water at room temperature.
8. Circle sections with Dako pen.
9. Wash in 20%fetal calf serum (FCS)/0.05M TBS pH7.6 for 5 minutes.
10. Apply CD31 antibody at 1:40 diluted in 20%FCS/TBS. Leave for one hour, then wash in TBS for 5 minutes.
11. Apply rabbit-anti mouse immunoglobulin diluted 1:50 in TBS for 30 minutes, then wash in TBS for 5 minutes.
12. Apply mouse APAAP diluted 1:100 in TBS for 30 minutes then wash in TBS for 5 minutes.
13. Re-apply rabbit anti-mouse immunoglobulin 1:50 in TBS for 10 minutes then wash in TBS for 5 minutes.
14. Re-apply mouse APAAP diluted 1:100 in TBS for 10 minutes, then wash in TBS for 5 minutes.
15. Apply new fuchsin substrate for 20 minutes.
16. Wash in distilled water.
17. Counterstain in Gill's no. 3 haematoxylin solution for 1-2 minutes with differentiation.
18. Rinse in running tap water.
19. Mount in aqueous mounting medium.

APAAP STAINING PROTOCOL FOR FACTOR VIII ANTIBODY.

The same protocol is used as for CD31, applying FVIII antibody 1:80 diluted in 20%FCS/TBS in place of CD31.

PROTOCOL FOR VEGF STAINING OF PARAFFIN EMBEDDED TUMOUR SECTIONS.

1. Mount 4-6μm thick tumour sections onto poly-L-lysine slides.
2. Deparaffinate slides as follows: xylene for 5 minutes x2 changes, absolute alcohol for 5 minutes x2 changes, 70% alcohol for 5 minutes x2 changes. Rinse sections in running water, then rinse in TBS.
3. Incubate sections in fresh 3% hydrogen peroxide for 5 minutes x2 changes to quench endogenous peroxidase activity. Rinse slides in running water, then TBS.
4. Incubate slides in normal goat serum diluted 1:10 in TBS for 30 minutes to block non-specific binding. Tap off serum and wipe away excess.
5. Incubate with primary antibody diluted 1:100 in TBS overnight at room temperature. Do not allow slides to dry out. Substitute TBS for primary antibody for the negative control.
6. Rinse in TBS three times and place in a TBS bath for 5 minutes.
7. Prepare secondary antibody (biotinylated goat anti-rabbit) as instructed in Dust kit and incubate for 30 minutes at room temperature. Repeat step 6.
8. Prepare StrepABComplex as instructed in Duet kit and incubate for 30 minutes at room temperature. Repeat step 6.
9. Incubate with DAB chromogenic substrate for 30 minutes at room temperature. Rinse in running water.
10. Counterstain lightly in haematoxylin for 3 minutes to stain cell nuclei.
11. Clear non-nuclear staining by incubating in acid alcohol for 2-3 minutes (0.5% in hydrochloric acid in 70% ethanol)
12. Incubate in water for 5-10 minutes at room temperature.
13. Dehydrate sections as follows: 70% alcohol for 5 minutes x2 changes, absolute alcohol for 5 minutes x2 changes, xylene for 5 minutes x2 changes.
14. Mount sections immediately in DPX and allow to dry before visualizing staining under a microscope. Cell staining positive for VEGF appear brown.

Appendix 2.3: ROUTINE SEQUENZA METHOD FOR STAINING WITH MIB-1 ANTIBODY.

1. Dewax paraffin slides (5 minutes) and take through alcohols to water.
2. Block endogenous peroxidase-3% hydrogen peroxide for 10 minutes. Wash in running water.
3. Perform antigen retrieval by microwaving sections in 1.07g. citric acid for 3x5minutes. Allow sections to stand in citric acid for 20 minutes. Rinse in tap water.
4. Load slides onto Sequenza. Apply PBS for 5 minutes.
5. Apply primary antibody: MIB-1 (Biogenex) 1:20 in 0.05M TBS for 30 minutes.
6. Apply PBS for 5 minutes.
7. Apply secondary antibody: Vector anti-mouse 1:200 in 0.05M TBS for 30 minutes.
8. Apply PBS for 5 minutes.
9. Apply tertiary reagent for 30 minutes.
10. Apply PBS for 5 minutes, followed by distilled water for 5 minutes.
11. Prepare DAB (2mls in 2µl hydrogen peroxide) and immerse sections in DAB for 5 minutes.
12. Rinse slides in tap water.
13. Counterstain with haematoxylin for 5 minutes. Differentiate in acid alcohol.
14. Dehydrate, clear in xylene and mount in DPX.

Appendix to Results.

1. Reproducibility of microvessel counts in breast cancer specimens.

Table A.1: Comparison of mvc performed by same observers at two time points.

Tumour no.	MVC1	MVC2
79	12	14
78	18	19
91	16	22
92	18	19
68	15	18
89	15	15
90	16	17
72	12	14
73	14	15
87	16	19
88	15	20
76	14	16
77	16	16
81	14	18
82	14	18
101	18	17
102	19	19
99	16	17
100	15	16
3	13	14
4	14	14
1	14	14
2	14	18
13	14	14
57	22	24
11543	15	18
46	15	18
26	17	15
12852	24	28
127	28	26
187	19	16

Table A1.2: Comparison of mvc performed by two observers.

Observers LM and KK

Tumour no.	MVC LM	MVC KK
408/97	27	27
24384/95	19	28
25637/95	18	19
7541/97	17	21
21126/95	15	13
3083/96	17	26
17564/96	14	12
23861/96	21	19
21767/96	22	21
25138/95	17	27
3084/96	17	19
21768/96	18	15
14247/96	18	25
20808/96	13	13
13636/95	17	18
2746/97	21	21

Table A1.3: Microvessel counts following staining with CD31 and FVIII antibodies on core biopsies and tumour sections.

SECTIONS			CORES		
Tumour No.	**MVC CD31**	**MVC FVIII**	**Tumour no.**	**MVC CD31**	**MVC FVIII**
4809/95	16	24	11253/96	16	16
282/92	19	10	247	17	17
12243/93	16	12	274	16	14
UB15222/91	26	21	6	13	16
3294/93	13	35	1A	13	12
5086/94	29	21	64	12	12
UB14022/91	11	19	9367/97	18	16
9286/92	12	17	275	14	13
919/94	22	15	196	14	17
10748/92	25	16	157	16	14
5432/94	14	17	6368/97	9	17
7435/92	22	19	115	12	18
1548/93	19	21	248	18	19
2767/93	35	18	136	9	10
466/94	21	23	293	12	10
960/95	28	26	94	15	15
9504/93	23	16	237	16	14
6640/95	23	19	11402/96	21	17
11247/92	17	17	249	16	14
10230/92	14	13	3987/97	15	14
3291/93	11	13	243	22	25
5383/92	25	21	12588/97	15	14
349/92	14	17	242	17	18
884/96	18	14	130	15	11
282/94	19	10	229	16	14
848/92	12	10	111	8	6
10750/92	19	18	8	17	12
UB19363/90	17	15	164	18	17
UB20711/91	18	23	279	17	16
885/96	19	17	287	20	16
4206/92	8	9	163	11	11
5372/92	22	16	277	14	15
2676/93	20	21	294	20	16
5851/95	18	13	117	13	14
9963/92	16	16	159	14	14
10749/92	11	11	PB2645/98	15	10
10708/94	18	16	271	14	14
7766/94	14	18		14	13
2653/94	13	17	250	11	13
7980/92	24	22	167	13	15
6221/92	15	12	131	20	14
3197/93	14	16	134	10	15
4982/93	17	13	32	10	14
9451/94	12	15	262	14	14
10781/93	11	18	291	14	18
5392/92	17	13	7	14	13
UB15071/91	15	23	1B	12	13

SECTIONS			CORES		
Tumour no.	MVC CD31	MVC FVIII	Tumour no.	MVC CD31	MVC FVIII
7057/95	10	16	206	16	15
5270/93	19	10	288	8	6
4684/93	23	18	208	11	12
2768/93	14	15	173	22	12
3977/93	19	15	160	17	12
89/93	20	12	122	21	12
849/92	14	12	261	13	14
2618/94	18	20	144	17	15
6093/92	15	15	301	14	12
5262/92	28	11	113	14	16
5176/94	6	15	238	13	14
436/92	24	26	34	11	21
9741/94	21	24	258	13	17
437/92	7	16	135	19	18
6291/94	16	11	256	17	19
10758/94	9	11	236	15	11
401/93	13	11	235	18	20
7618/93	12	14	145	13	12
5178/94	12	21	232	12	12
6082/95	11	12	116	12	16
2475/95	25	21	10	19	16
2652/94	14	19	172	9	18
11532/95	21	15	290	16	17
4773/96	27	28	295	16	18
6930/96	28	27	179	9	16
2487/93	23	8	289	15	14
10584/92	26	18	298	22	15
6240/92	12	15	133	10	9
8388/95	20	32	171	12	16
5247/96	23	25	296	14	18
5483/94	18	12	273	17	19
10812/96	26	14		13	22
6239/92	15	15	265	20	16
3523/93	12	16	182	10	19
UB27088/90	27	19	147	19	18
850/92	22	19	155	8	11
5991/96	11	16	302	10	10
2834/93	11	10	311	6	14
10263/92	18	15	1C	12	14
9484/94	19	26	309	13	13
9654/95	10	9	214	12	13
2292/93	12	15	300	16	18
9656/95	18	15	195	13	13
1672/95	10	11	320	10	13
2855/95	17	22	165	10	10
1365/93	23	17	304	13	18
9739/94	0	18	194	11	10
7927/94	19	17	284	17	16
5917/95	9	22	314	13	14
8845/92	13	11	315	15	12
2819/94	24	21	312	13	16

SECTIONS			CORES		
Tumour no.	**MVC CD31**	**MVC FVIII**	**Tumour no.**	**MVC CD31**	**MVC FVIII**
11971/95	13	17	313	0	0
10293/93	10	13	308	18	18
10063/93	14	14	307	18	15
8384/93	11	12	215	13	12
6141/93	20	20	220	13	17
4485/92	11	17	1	14	12
8114/96	18	18	68	18	19
2037/95	13	20	19	21	21
1831/96	7	15	91	16	15
435/92	14	21	14	17	20
2	18	19	23	13	14
69	18	18	99	17	14
20	18	21	101	17	18
92	19	14	79	14	16
10547	18	20	87	19	20
102	19	19	81	18	15
78	19	22	76	16	15
88	20	18	3	14	14
82	18	17	72	14	13
77	16	15	89	15	13
40	19	13	13	14	18
4	14	15			
73	17	13			
90	17	20			
10548	16	16			
4100/97	20	18			
141	16	13			
142	19	13			
266	18	22			
268	15	20			
230	13	13			
188	19	18			
299	17	17			
143	16	18			
10846/97	13	18			
70	21	19			
244	22	11			
8516/97	14	16			
5115/97	15	16			
4215/97	22	22			
305	20	16			
222	23	13			
186	15	21			
280	16	14			
231	13	23			

Table A1.4: Counts performed in core biopsies and tumour sections taken simultaneously.

Tumour no.	No. cores	MVC of cores	Tumour no.	MVC of section
1	2	14	2	18
68	6	18	69	18
19	3	21	20	18
91	4	16	92	19
56	3	18	57	24
14	2	17	10547	18
99	2	17	100	16
101	2	17	102	19
79	1	14	78	19
87	7	19	88	20
81	3	18	82	18
76	3	16	77	16
3	4	14	4	14
72	4	14	73	17
89	3	15	90	17
13	5	14	10548	16

Table A1.5: Comparison of counts performed on core biopsies and later tumour sections.

Tumour no.	No. cores	MVC of cores	LM No. 2	MVC of section
223	2	19	12852	15
181	3	20	8972	22
51	2	18	69	18
41	4	15	57	22
11543	6	18	27	18
110	2	17	4785	25
55	3	13	82	21
207	3	20	210	18
59	2	21	88	16
83	3	16	2455	25
170	3	14	8791	23
86	2	16	3145	24
28	1	11	310	17
46	3	13	66	22
112	5	25	127	20
96	3	12	3073	17
11708	3	21	12025	21
26	1	19	102	19
107	4	15	187	22
264	2	16	267	21
128	5	14	140	16

Table A1.6: Comparison of mvc performed on core biopsies of same tumour at separate times.

Tumour no. cores 1	No. cores 1	MVC of cores 1	Tumour no. cores 2	No. cores 2	MVC of cores 2
41	4	15	56	4	18
51	2	18	68	6	18
55	3	13	81	3	18
59	2	21	87	7	18
317	2	18	6525	2	14
316	3	17	5816	2	19
318	2	20	6334	4	15
319	2	16	6643	1	21
321	2	10	6418	3	20
322	3	16	7095	4	13

Table A1.7: Relationship between number of cores and % change in mvc.

Number of cores	MVC1 of cores	MVC difference MVC1-MVC2	% MVC difference
1	14	-5	35.7
1	11	6	54.5
1	19	0	0
2	14	-4	28.6
2	17	-1	5.88
2	17	1	5.88
2	17	-2	11.8
2	19	4	21.1
2	18	0	0
2	17	8	47.1
2	21	-5	23.8
2	16	8	50
2	16	5	31.2
3	21	3	14.3
3	18	-6	33.3
3	18	0	0
3	16	0	0
3	15	-2	13.3
3	20	-2	10
3	13	7	53.9
3	20	-2	10
3	16	9	56.3
3	14	9	64.3
3	13	9	69.2
3	12	5	41.7
3	21	0	0
4	16	-3	18.8
4	14	0	0
4	14	-3	21.4
4	15	7	46.7
4	15	7	46.7
5	14	-2	14.3
5	25	-5	20
5	14	2	14.3
6	18	0	0
6	18	0	0
7	19	-1	5.3

2. Changes in tumour vascularity following primary tamoxifen treatment and correlation with response.

Table A2.1: Tumour volume during treatment with tamoxifen.

Where method of assessment is US: ultrasound, CLIN: clinical, MAMM: mammography.

Patient no.	Clin. size 1	US size 1	Tum vol 1	Clin. size 2	US size 2	Tum vol 2	Clin. size 3	US size 3	Tum vol3	Assess.
480425W	37x35x38	11x13x17	1256	37x35x33	14x11x11	875	32x28x34	13x14x13	1222	US
495639K	40x38	NS	NS	34x38	26	9203	32x32	19x16	2865	US
558680X	32x31x30	15x13x22	2217	34x30x29	17x13x14	1577	24x28x25	23x18x11	2277	US
495980E	NS	NS	NS	NS	NS	NS	NS	NS	NS	CLIN
494064R	54x35	NS	NS	52x33	NA	NS	56x41	NS	NS	CLIN
545602E	58x58	NS	102160	59x60x50	NS	92677	56X53X53	19x16	82364	CLIN
582565W	35x38x34	21x8x10	1890	32x33x29	21x17x18	3213	30x38x31	20x19x17	3383	US
528456W	NS	min. red.		NS	min. red.		NS	NS		US
572868X	55x62	48x28x27	19000	57x65	30x33x27	13996				US
596319W	50x40x45	39x26x36	18252	32x37x34	33x24x31	24552	35x31	38x24x32	14592	US
522679X	47x47	NS	NS	NS	NS	NS	NS	NS	NS	CLIN
502246A	49	NS	NS	52x48x50	NS	NS	50x47x50	39x38x32	NS	CLIN
524732W	NS	NS	NS	NS	NS	NS	NS	NS	NS	MAM MO
524111X	NS	NS	NS	23x25x26	16x14x6	704	26x23x26	15x14x7	770	US
268300H	45x60x53	28x13x17	3240	45x45x55	14x15x18	1979	20x20	10x12x12	754	US
110258V	37	22x15x18	3110	30	NS	NS	NS	13x10x11	749	US
528171K	26x28x29	17x16x18	2330	NS	17x11x15	1470				US
552168H	35x39	20x16x18	3016	28x30x31	19x16x15	2388	NS	12x13x12	1062	US
52346X	45	NS	NS	46	NS		41	NS		MAM MO
560577L	19X24X17	17X13X11	1273	14X20X12	12X9X12	679	14X15X18	8X9X9		US
513260L	32	17X12.5		NS	NS		NS	NS		CLIN
527028L	26	NS	NS	NS	10	524	NS	7.5x6	165	US
44955M	34x36	NS	NS	26x32	15x17x13	1736				CLIN
395041K	44X38X39	17X23X29	5937	30X30	13X20X19	2587	40X35X39	21X14X25	1200	US
350429H	43x41x45	22x16x23	5280	40x31x28	21x16x23	3864	36x32x31	15x10x14	1050	US
545123B	30x42x38	37x39x15	11333	32x37x37	28x26x12	8736	27x23x20	7x15x18	990	US
520042V	NS	NS	NS	NS	NS	NS				CLIN
81364H	36	NS		30X28	static		23x22	NS		MAM MO
511159M	NS	NS	NS	33	20x12	NS	33	NS	NS	CLIN
500371K	26	NS	NS	20	NS					CLIN
372859M	30x35x34	14x19x23	3203	35x25x22	19x17x22	3721	30x28	16x17x21	2991	US
529249W	NS	NS	NS	24X22	NS	NS	NS	NS		CLIN
376029R	25X26X34	18X18X12	2036	35X30X23	NS	NS	27X25X27	10X19X20	1990	US
499870K	44x38	NS	NS	40X37	NS		40X40	NS		US
520767V	NS	NS	NS	28x18	NS		NS	NS		CLIN
498214W	NS	15x14x15	1649	NS	15x9x13	919	NS	16x3x10	251	US
498258R	NS	30x21	8247	38	34x37x42	27665	31x35	26x19x23	5949	US
559968K	41x45x36	27x32x18	8143	29x35x35	30x23x13	4697	25x28x30	11x19x31	3392	US
510657M	NS	NS	NS	NS	NS	NS	NS	NS	NS	CLIN
450908A	40x38x30	17x15x27	3605	13x16x!5	40x34x34	1634	13x12x16	32x30x28	1307	US
155271L	36x34x35	14x16x18	2111	37x30x29	13x14x17	1620	20x20	NA		US
519014W	NS	NS	NS	NS	NS	NS	NS	NS	NS	CLIN
499532M	38x40x38	17x18x16	2564	35x34x35	15x14x11	1210	34x32x29	11x13x12	899	US
564461M	34x32x29	12x12x14	1056	34x30x27	NA		30	NA		CLIN
515126A	NS	NS	NS	NS	NS	NS	30	NS	NS	CLIN
529146X	49X33X51	NS	NS	44X48X48	NS		33X34X37	NS	NS	MAM MO
458317M	47x44x38	25x23x27	11140	35x30x25	18x15x19	2686	38x35x42	10x23x25	3011	US
548395L	46x51x46	32x34x21	11963	43x44x40	22x17x31	6071				US
509362K	NS	reduced					excellent			CLIN
503527E	NS	static		35x37	NS		reduction			CLIN
526825K	NS	NS	NS	NS	NS	NS				CLIN
488627M	45x50x44	33x29x20	10022	33x35x27	20x10x14	1047	31x31x29	NA		US
535168M	NS	NS	NS	NS	NS	NS	NS	22x30		CLIN
532363A	NS	NS	NS	NS	NS	NS	NS	NS	NS	CLIN
527259E	34	NS	NS	NS	NS	NS	NS	NS	NS	CLIN
527857A	NS	NS	NS	NA	12x9x11	622	NS	NS	NS	CLIN
588347R	38x40x37	16x17x17	2421	35x34	16x17x17	2421	25x25	15x12x13	1225	US

Microvessel counts following staining with antibody to Factor VIII.

Table A1.2: Pre-and post-treatment mvc following staining with antibody to Factor VIII for all tumours.

Patient no.	Pre-treatment mvc	Post-treatment mvc	Patient no.	Pre-treatment mvc	Post-treatment mvc
480425W	12	15	**81364H**	13	10
495639K	21	26	**511159M**	21	18
558680X	21	24	**500371K**	17	15
495980E	19	16	**372859M**	19	32
494064R	21	19	**529249W**	18	9
545602E	16	17	**376029R**	10	12
582565W	19	15	**499870K**	10	15
528456W	13	19	**520767V**	18	16
572868X	16	27	**498214W**	15	19
596319W	14	25	**498258R**	23	19
522679X	12	20	**559968K**	17	16
502246A	12	17	**510657M**	16	15
524732W	18	18	**450908A**	13	9
524111X	19	20	**155271L**	13	9
268300H	24	7	**519014W**	16	15
110258V	10	11	**499532M**	24	22
528171K	35	18	**564461M**	18	11
552168H	15	11	**515126A**	22	17
52346X	16	18	**529146X**	16	27
560577L	17	11	**458317M**	24	18
513260L	19	11	**548395L**	18	21
527028L	18	14	**509362K**	13	11
44955M	23	21	**503527E**	23	12
395041K	26	12	**526825K**	11	7
350429H	25	21	**488627M**	16	17
545123B	16	19	**535168M**	10	13
520042V	13	8	**532363A**	18	14
588347R	15	15	**527259E**	15	12
			527857A	15	23

Table A2.3: Comparison of pre-and post-treatment mvc in responding tumours:

Pre-treatment mvc	Post-treatment mvc
24	7
10	11
35	18
15	11
16	18
17	11
19	11
18	14
23	21
26	12
25	21
16	19
13	8
13	10
21	18
17	15
19	32
18	9
10	12
10	15
18	16
15	19
23	19
17	16
16	15
13	9
13	9
16	15
24	22
18	11
22	17
16	27
24	18
18	21
13	11
23	12
11	7
16	17
10	13
18	14
15	12
15	23
15	15

Table A2.4: Comparison of pre-and post-treatment mvc in non-responding tumours.

Pre-treatment mvc	Post-treatment mvc
12	15
21	26
21	24
19	16
21	19
16	17
19	15
13	19
16	27
14	25
12	20
12	17
18	18
19	20

Table A2.5: % reduction in tumour volume and change in mvc with treatment in responding tumours:

RESPONDERS	
% tumour volume reduction	%mvc change (mvc2-mvc1/mvc1%)
92	-71
72	10
28	-48.6
48	12.5
73	-35.3
88	-42
91	-17.4
80	-54
80	-16
91	18.75
43	-38.5
53	-15.4
45	-14.3
78	-11.8
45	68.5
67	-50
40	20
29	50
49	-11
87	26.7
32	-17.4
83	-5.88
72	-6.25
64	-30.8
30	-31
55	-6.25
65	-8.33
64	-39
32	-22.7
85	68.75
73	-25
84	16.7
41	-15.4
98	6.25
68	-22.2
61	-20
49	0

Table A2.6: % reduction in tumour volume and change in mvc with treatment in non-responding tumours:

NON-RESPONDERS	
% tumour volume reduction	**%mvc change (mvc2-mvc1/mvc1%)**
3	25
0	24
0	14
0	-16
0	-9.5
0	6.25
0	-21
18	46
13	68.75
21	78.6
0	66.7
0	41.7
0	0
4	0

Microvessel counts following staining with antibody to CD31.

Table A2.7: Pre- and post-treatment mvc in all tumours following staining with antibody to CD31:

Patient no.	Pre-treatment mvc	Post-treatment mvc	Patient no.	Pre-treatment mvc	Post-treatment mvc
480425W	16	6	**511159M**	25	26
495639K	26	24	**500371K**	14	12
558680X	29	21	**372859M**	0	20
495980E	11	7	**529249W**	0	0
494064R	19	0	**376029R**	19	18
545602E	0	0	**499870K**	12	15
582565W	23	21	**520767V**	19	12
528456W	0	0	**498214W**	17	27
572868X	0	28	**498258R**	18	22
596319W	18	23	**559968K**	19	11
522679X	20	20	**510657M**	22	18
502246A	14	11	**450908A**	0	0
524732W	0	18	**155271L**	18	10
524111X	0	0	**519014W**	16	12
268300H	16	0	**499532M**	0	0
110258V	19	28	**564461M**	14	10
528171K	13	0	**515126A**	24	23
552168H	22	16	**529146X**	14	0
52346X	25	0	**458317M**	0	0
560577L	14	9	**548395L**	11	24
513260L	22	13	**509362K**	17	13
527028L	35	12	**503527E**	15	8
44955M	21	12	**526825K**	20	0
395041K	28	11	**488627M**	10	13
350429H	0	25	**535168M**	19	10
545123B	23	14	**532363A**	23	14
520042V	14	23	**527259E**	14	11
81364H	11	0	**527857A**	19	0
			588347R	0	7

Table A2.8: Comparison of pre-and post-treatment mvc in responding tumours:

Pre-treatment mvc	Post-treatment mvc
16	0
19	28
13	0
22	16
25	0
14	9
22	13
35	12
21	12
28	11
0	25
23	14
14	23
11	0
25	26
14	12
0	20
0	0
19	18
12	15
19	12
17	27
18	22
19	11
22	18
0	0
18	10
16	12
0	0

Table A2.9: Comparison of pre- and post-treatment mvc in non-responding tumours:

Pre-treatment mvc	Post-treatment mvc
16	6
26	24
29	21
11	7
19	0
0	0
23	21
0	0
0	28
18	23
20	20
14	11
0	18
0	0

Table A2.10: % reduction in tumour volume and change in mvc with treatment in responding tumours:

% tumour volume reduction	%mvc change (mvc2-mvc1/mvc1%)
92	-100
72	47.4
28	-100
48	-100
73	-35.7
87.7	-41
91	-66
80	-61
80	0
91	-39
43	64.3
53	-100
45	4
78	-14
45	0
67	0
40	-5.3
29.1	25
49	-37
87	59
32	22
83	-42
72	-18
64	0
23	-44
55	-25
65	0
64	-29
31.9	-4.2
85	-100
73	0
84	119
41	-24
98	30
68	-39
61	-21
49	0

Table A2.11: % reduction in tumour volume and change in mvc with treatment in non-responding tumours:

% tumour volume reduction	%mvc change (mvc2-mvc1/mvc1%)
3	-62.5
0	-8
0	-27.6
0	-36.4
0	-100
0	0
0.5	-10
18	0
13	0
21	28
0.5	-4
0	-21
0	5
4	0

Table A2.12: Comparison of counts following staining with Factor VIII and CD31:

Pre-treatment mvc Factor VIII	Pre-treatment mvc CD31	Post-treatment mvc Factor VIII	Post-treatment mvc CD31
12	16	15	6
21	26	26	24
21	29	24	21
19	11	16	7
21	19	19	0
16	0	17	0
19	23	15	21
13	0	19	0
16	0	27	28
14	18	25	23
12	20	20	20
12	14	17	11
18	0	18	18
20	18	20	13
24	16	7	0
10	19	11	28
35	13	18	0
15	22	11	16
16	25	18	0
17	14	11	9
19	22	11	13
18	35	14	12
23	21	21	12
26	28	12	11
25	0	21	25
16	23	19	14
13	14	8	23
13	11	10	0
21	25	18	26
17	14	15	12
19	0	32	20
18	0	9	0
10	19	12	18
10	12	15	15
18	19	16	12
15	17	19	27
23	18	19	22
17	19	16	11
16	22	15	18
13	0	9	0
13	18	9	10
16	16	15	12
24	0	22	0
18	14	11	10
22	24	17	23

Pre-treatment mvc Factor VIII	Pre-treatment mvc CD31	Post-treatment mvc Factor VIII	Post-treatment mvc CD31
16	14	27	0
24	0	18	0
18	11	21	24
13	17	11	13
23	15	12	8
11	20	7	0
16	10	17	13
10	19	13	10
18	23	14	14
15	14	12	11
15	19	23	0
15	0	15	7

Table A2.13: Percentage lymph node involvement and tumour response to tamoxifen:

Percentage lymph node involvement in non-responders	Percentage lymph node involvement in responders
0	0
44	0
0	0
0	25
100	0
0	0
0	33
77	0
6	0
84	47
0	7
100	0
	25
	13
	64
	0
	0
	57
	13
	46
	0
	6
	0
	22
	0
	0
	0
	0
	17
	42
	0
	12
	0
	0
	25
	17
	0
	0
	29

Table A2.14: Microvessel counts and % lymph node involvement in responding tumours: Lymph node status=number of nodes involved/number excised.

Pre-treatment mvc (mvc1)	Post-treatment mvc (mvc2)	mvc difference (mvc2-mvc1)	Lymph node status	% Lymph node involvement
24	7	-17	0/18	0
35	18	-17	0/4	0
16	18	2	0/4	0
17	11	-6	1/4	25
19	11	-8	0/5	0
18	14	-4	0/2	0
23	21	-2	5/15	33
26	12	-14	0/5	0
25	21	-4	0/16	0
16	19	3	7/15	47
13	8	-5	1/14	7
13	10	-2	0/9	0
21	18	-3	1/4	25
17	15	-2	2/15	13
19	32	13	16/25	64
18	9	-9	0/6	0
10	15	5	0/4	0
18	16	-2	8/14	57
15	19	4	1/8	13
23	19	-4	11/24	46
17	16	-1	0/3	0
16	15	-1	1/18	6
13	9	-4	0/6	0
13	9	-4	2/9	22
16	15	-1	0/10	0
24	22	-2	0/4	0
18	11	-7	0/34	0
22	17	-5	0/4	0
16	27	11	1/6	17
24	18	-6	5/12	42
18	21	3	0/4	0
13	11	-2	2/17	12
23	12	-11	0/16	0
11	7	-4	0/16	0
10	13	3	1/4	25
18	14	-4	3/18	17
15	12	-3	0/3	0
15	23	8	0/2	0
15	15	0	5/17	29
19	20	1	0/4	0

Table A2.15: Microvessel counts and % lymph node involvement in non-responding tumours:

Pre-treatment mvc (mvc1)	Post-treatment mvc (mvc2)	mvc difference (mvc2-mvc1)	Lymphnode status	% lymph node involvement
12	15	3	0/5	0
21	26	5	7/16	44
21	24	3	0/6	0
19	16	-3	0/15	0
21	19	-2	23/23	100
16	17	1	0/25	0
13	19	6	0/4	0
16	27	11	10/13	77
14	25	11	1/18	6
12	20	8	16/19	84
12	17	5	0/4	0
18	18	0	6/6	100

Table A2.16: Level of ER expression and response:

ER in fmol/mg cytosolic protein.

ER level in non-responding tumours	ER level in responding tumours
140	324
113	606
398	325
29	363
87	605
109	254
132	444
68	445
33	42
673	221
40	379
201	288
465	620
25	186
	510
	72
	33
	964
	96
	262
	463
	53
	837
	58
	557
	739
	1455
	25
	546
	51
	76
	1292
	166
	762
	623
	166
	386
	197
	292
	1496
	306
	1388
	357

Table A2.17: Microvessel counts and level of ER expression in responding tumours:

Pre-treatment mvc (mvc1)	mvc difference (mvc2-mvc1)	%mvc change (mvc2-mvc1/ mvc1%)	ER level
24	-17	-70.8	324
10	1	10	606
35	-17	-48.6	325
15	-4	-26.7	363
16	2	12.5	605
17	-6	-35.3	254
19	-8	-42.1	444
18	-4	-22.2	445
23	-2	-8.7	42
26	-14	-54	221
25	-4	-16	379
16	3	18.75	288
13	-5	-38.5	620
13	-2	-15.4	186
21	-3	-14.3	510
17	-2	-11.8	72
19	13	72	33
18	-9	-50	964
10	2	20	96
10	5	50	262
18	-2	-11	463
15	4	26.7	53
23	-4	-17.4	837
17	-1	-5.9	58
16	-1	-6.25	557
13	-4	-30.8	739
13	-4	-30.8	1455
16	-1	-6.25	25
24	-2	-8.33	546
18	-7	-38.9	51
22	-5	-22.7	76
16	11	68.75	1292
24	-6	-25	166
18	3	16.7	762
13	-2	-15.4	623
23	-11	-47.8	166
11	-4	-36.4	386
16	1	6.25	197
10	3	30	292
18	-4	-22.2	1496
15	-3	-20	306
15	8	53	1388
15	0	0	357

Table A2.18: Microvessel counts and level of ER expression in non-responding tumours:

Pre-treatment mvc (mvc1)	mvc difference (mvc2-mvc1)	%mvc change (mvc2-mvc1 /mvc1%)	ER level
12	3	25	140
21	5	23.8	113
21	3	14.3	398
19	-3	-15.8	29
21	-2	-9.5	87
16	1	6.25	109
19	-4	-21	132
13	6	46.2	68
16	11	68.75	33
14	11	78.6	673
12	8	66.7	40
12	5	41.7	201
18	0	0	465
19	0	0	25

4. Sequential changes in tumour vascularity during treatment with tamoxifen: a prospective study.

Table A4.1: Tumour response data for patients treated with tamoxifen (where method of assessment is classified as US: ultrasound, CLIN: clinical or mammo: mammographic)

Patient no.	Clinical size1	US size1	Clinical size2	US size2	Clinical size3	US size3	Mammo	Mammo resp.	Assess.
615724L	50x60	43x50	50x65	43x50	55x53	53x46	30397	sl decr.	US
639991L	41x28 (ulcer)	NA	42x34	NA	41x28 (ulcer)	NA	NA		CLIN
120288B	38x38	21x17x19	30x30	21x16x18	30x30	18x16x15	150698	static	US
612031H	41x40	NA	34x32x36	NA	30x27x28	NA	NA		CLIN
614905K	24x22x26	24x22x23	23x21x25	16x11x17	14x10	14x11x17	100297	decrease	US
614905K	70x68x72	53x21x25	68x66x70	62x51x27	66x68x70	43x16x35	300597	decrease	US
296162H	48x38	28x26x24 NA		28x26x24	38x28	28x28x26	80198	increase	US
631385B	28x35	27x21x19	24x26	25x15x17	25x24	27x16x15	150698	sl.red.	US
143050M	40x40	25x15x20	35x25	27x22x27	33x22	13x27x25	40298	reduction	US
628989R	25x20	20x11x15	25x20	19x11x18	15x20	13x11x9	221097	reduction	US
495945A	24x22x26	26x15x21	26x27	26x20x13	20x25	16x11x21	31197	reduction	US
623397B	25x25	25x22x21	23x25	18x19x18	33x25	22x14x12	200897	reduction	US
561829A	59x58	31x40x22	54x55	17x60x14	40x40	20x33x24	NA		US
341122W	35x40	29x13x23	35x45	31x29x14	30x32	25x18x17	NA		US
645382X	29x29	20x13x19	29x29	20x13x19	30X27	15X18X8	NA		US
622149X	NA	43x40x20	45x25	43x24x20	NA	27x19x13	150797	reduction	US
D345548	34x30	23x20x12	32x30	21x20x19	28x22	18x13x10	30398	reduction	US
614975H	40x40	24x26x25	35x35	23x20x17	impalp.	20x18x15	140597	reduction	US
640026R	50x37	28x27x30	47x42	27x23x30	30x35	20x18x24	110598	static	US
624169K	20x20	34x13x18	30x25	34x13x18	30x25	34x13x18	180797	static	US
639575E	46x28	19x34x18						reduction	mammo
638318X	22x22	18x28x18	28x33	20x24x13	25x16	19x9x20	90498	static	US
637345V	45x30	18x16x29	37x24	15x20x15	26x19	24x9x16	200498	reduction	US
558811X	52x35	32x47x20	50x42	35x23x37	52x52	43x39x18	NA	NA	US
637357K	40x48	25x18x28	40x32	19x19x15	43x35	20x22x18	160398	static	US
422318K	30x28x25	NA	24x22x20	NA	28x30x32	NA	10997	increase	CLIN
84321A	25x27x23	NA	18x20x19	16X20	16x19x18	NA	290197	reduction	CLIN
628483V	NA	24x18x17	45x50	24x31x18	38x35	26x18x17	31197	static	US
D077978	42x45	19x20x13	34x40	15x14x14	30x38	19x11x17	NA	NA	US
643001M	32x32	33x15x17	30x20	33x12x10	NA	33x12x10	230798	not seen	US
629111W	42x34	25x12	42x35	39x26x23	30x25	25x10x22	131097	static	US
484933K	17x21	15x16x11	16x17	15x7x12	16x16	11x6.2	NA		US
266308W	32x33	24x33x25	26x26	17x19x16	22x24	19x20x13	290698	reduction	US
622926V	NA	30x44x18	NA	26x16x28	NA	14x9x14	NA	NA	US
625368K	30x30	20x15x11	30x33	29x14x16	30x25	20x22x11	20298	static	US
D528581	19x23	15x24x13	11x26	12x8x11	12x14	11x14x13	NA		US
10046V	41x50	22x24x29	40x41	22x11x21	36x35	21x11x15	150698	reduction	US
								static	mammo
201525A	35x33	28x10x19	30x25	30x12x20	31x28	20x19x14	60598	static	US
629740R	45x55	28x27x17	35x32	22x16x9	22x30	18x14x7	61197	reduction	US
625691A	40x45	27x41x15	35x45	35x38x22	45x55	30x26x17	41197	reduction	US
626230E	35x35	38x18x29	34x30	31x15x17	35x20	25x18x11	NA		US

Microvessel counts following staining with antibody to Factor VIII.

Table A4.2: Pre-, peri-and post-treatment mvc in all patients:

Patient no.	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
615724L	16	14	18
639991L	17	14	10
120288B	14	18	14
612031H	16	13	13
614905K	12	13	14
614905K	12	17	13
296162H	16	15	22
631385B	13	6	13
143050M	17	12	20
628989R	14	12	13
495945A	17	12	13
623397B	18	12	18
561829A	19	14	18
341122W	10	15	13
645382X	10	12	13
622149X	15	16	10
D345548V	14	14	17
614975H	17	21	18
640026R	14	17	18
624169K	14	18	16
639575E	25	19	16
638318X	14	11	22
637345V	18	20	16
558811X	11	12	10
637357K	14	12	16
422318K	6	16	18
84321A	12	16	19
628483V	17	18	11
D077978R	16	17	14
643001M	16	18	12
629111W	11	16	13
484933K	15	14	20
266308W	16	15	16
622926V	14	9	21
625368K	14	16	14
D528581B	10	18	0
10046V	14	19	18
	13	22	9
201525A	13	16	15
629740R	15	19	23
625691A	14	18	12
626230E	15	11	17

Table A4.3: Pre-, peri- and post-treatment mvc in non-responding and responding tumours:

(Non-responding tumours in **bold** type)

Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
16	**14**	**18**
17	**14**	**10**
14	**18**	**14**
16	**13**	**13**
12	**13**	**14**
12	**17**	**13**
16	**15**	**22**
13	**6**	**13**
17	**12**	**20**
14	**12**	**13**
17	**12**	**13**
18	**12**	**18**
19	**14**	**18**
10	**15**	**13**
10	12	13
15	16	10
14	14	17
17	21	18
14	17	18
14	18	16
25	19	16
14	11	22
18	20	16
11	12	10
14	12	16
6	16	18
12	16	19
17	18	11
16	17	14
16	18	12
11	16	13
15	14	20
16	15	16
14	9	21
14	16	14
10	18	0
14	19	18
13	22	9
13	16	15
15	19	23
14	18	12
15	11	17

Table A4.4: Relationship between degree of ultrasound response and % mvc change with tamoxifen.

(% mvc change=mvc3-mvc1/mvc1% where mvc3 is post-treatment mvc and mvc1 is pre-treatment mvc) Non-responding tumours are shown in **bold type.**

% tumour volume reduction	% change in mvc
-21	**12.5**
14	**8.3**
-17	**37.5**
-8	**17.7**
19	**30**
0	**14.3**
0	**-9.1**
0	**-35.3**
12	**-12.5**
8	**18.18**
0	**15.4**
20	**-14.4**
36	0
	-18.75
78	-14.3
59	0
67	-7.1
55	-23.5
41	0
42	-5.6
50	30
77	-33
58	21.4
50	5.88
66	28.6
	-36
49	57
59	-11.1
36	14.3
	58
48	-25
46	33.3
75	0
93	50
25	0
46	-100
80	28.6
87	53.3
75	13.3

Microvessel counts following staining with antibody to CD31.

Table A4.5: Pre-, peri-and post-treatment mvc in all patients:

Patient no.	Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
615724L	16	10	20
639991L	17	14	10
120288B	16	14	6
612031H	13	14	16
614905K	13	12	12
614905K	12	15	19
296162H	18	16	18
631385B	14	8	13
143050M	14	11	15
628989R	16	22	13
495945A	9	17	12
623397B	12	21	19
561829A	18	13	16
341122W	9	17	13
645382X	12	14	10
622149X	15	14	10
D345548V	16	13	17
614975H	21	11	16
640026R	16	13	13
624169K	15	19	14
639575E	22	17	15
638318X	15	15	22
637345V	17	18	20
558811X	15	13	11
637357K	16	12	17
422318K	8	12	13
84321A	17	19	21
628483V	18	9	22
D077978R	17	16	13
643001M	20	16	15
629111W	11	9	23
484933K	14	15	15
266308W	20	22	13
622926V	13	10	15
625368K	14	12	16
D528581B	15	14	0
10046V	14	17	18
	14	13	12
201525A	11	20	18
629740R	13	10	13
625691A	20	19	13
626230E	10	8	13

Table A4.6: Comparison of microvessel counts in non-responding and responding tumours before, during and after treatment with tamoxifen.

(Non-responding tumours shown in **bold** type)

Pre-treatment mvc	Peri-treatment mvc	Post-treatment mvc
16	**10**	**20**
17	**14**	**10**
16	**14**	**6**
13	**14**	**16**
13	**12**	**12**
12	**15**	**19**
18	**16**	**18**
14	**8**	**13**
14	**11**	**15**
16	**22**	**13**
9	**17**	**12**
12	**21**	**19**
18	**13**	**16**
9	**17**	**13**
12	14	10
15	14	10
16	13	17
21	11	16
16	13	13
15	19	14
22	17	15
15	15	22
17	18	20
15	13	11
16	12	17
8	12	13
17	19	21
18	9	22
17	16	13
20	16	15
11	9	23
14	15	15
20	22	13
13	10	15
14	12	16
15	14	0
14	17	18
14	13	12
11	20	18
13	10	13
20	19	13
10	8	13

Table A4.7: % reduction in tumour volume and % change in mvc with treatment (% mvc change=mvc3-mvc1/mvc1%, where mvc1=pre-treatment mvc and mvc3=post-treatment mvc. Non-responding tumours are shown in **bold type**).

% reduction in tumour volume	% change in microvessel count with treatment
-21	**25**
14	**58**
-17	**0**
-8	**7.1**
19	**44**
0	**-6.7**
0	**-26.7**
0	**22.2**
12	**-23.5**
8	**109**
0	**63.6**
20	**-35**
36	-62.5
	23.1
78	-7.7
59	-7.1
67	-18.75
55	33.3
41	58.3
42	-11.1
50	-16.7
77	-33.3
58	6.25
50	-23.8
66	-18.75
	-31.8
49	46.7
59	17.6
36	6.25
	23.5
48	-25
46	7.1
75	-35
93	15.4
25	14.3
46	-100
80	28
87	0
75	30

Microvessel counts and other tumour parameters.

Table A4.8: % lymph node involvement and tumour response to tamoxifen

% lymph node involvement in non-responders	% lymph node involvement in responders
77	25
100	0
0	0.2
0.25	0.4
5.3	0.5
100	12.5
0.4	50
36	0.1
44	18
	61

Table A4.9: Microvessel counts and lymph node involvement (Factor VIII):

Non-responders in **bold** type

Pre-treatment mvc (mvc1)	Post-treatment mvc (mvc3)	Change in mvc (mvc3-mvc1)	% mvc change	% lymph node involvement
16	**18**	**2**	**12.5**	77
12	**13**	**1**	**8.33**	**100**
16	**22**	**6**	**37.5**	**0**
17	**20**	**3**	**17.7**	**0**
14	**16**	**2**	**14.3**	**53**
11	**10**	**-1**	**-9.09**	**100**
6	**18**	**12**	**200**	**0**
17	**11**	**-6**	**-35.2**	**36**
11	**13**	**2**	**18.2**	**44**
16	13	-3	-18.75	25
14	13	-1	-7.14	0
18	18	0	0	0
17	18	1	5.88	0
14	22	8	57.1	0
18	16	-2	-11.1	12.5
12	19	7	58.3	50
14	21	7	50	0
14	14	0	0	18
15	23	8	53.3	61

Table A4.10: Microvessel counts and lymph node involvement (CD31 staining):

Non-responders in **bold** type

Pre-treatment mvc (mvc1)	Peri-treatment mvc (mvc2)	Post-treatment mvc (mvc3))	Change in mvc (mvc3-mvc1)	% mvc change	% lymph node involvement
16	**10**	**20**	**4**	**25**	**77**
12	**15**	**19**	**7**	**58**	**100**
18	**16**	**18**	**0**	**0**	**0**
14	**11**	**15**	**1**	**7.1**	**0**
15	**19**	**14**	**-1**	**-6.7**	**5.3**
15	**13**	**11**	**-4**	**-26.7**	**100**
8	**12**	**13**	**5**	**62.5**	**0**
18	**9**	**22**	**4**	**22**	**36**
11	**9**	**23**	**12**	**109**	**44**
13	14	16	3	23	25
16	22	13	-3	-18.8	0
12	21	19	7	58	0
21	11	16	-5	-23.8	0
15	15	22	7	46.7	0
17	18	20	3	17.7	12.5
17	19	21	4	23.5	50
13	10	15	2	15.4	0
14	12	16	2	14.3	18
13	10	13	0	0	61

Table A4.11: Level of ER expression (Histoscore) **and response:**

ER level in non-responding tumours	ER level in responding tumours
250	280
270	120
230	270
300	300
270	270
300	260
300	300
280	300
170	300
165	300
300	280
230	145
300	280
	270
	300
	270
	260
	300
	290
	260
	280
	300
	270
	300
	210
	290
	220
	290

Table A4.12: Pre-treatment mvc and level of ER expression (Factor VIII staining)

Non-responders in **bold** type:

Pre-treatment mvc	Level of ER expression (Histoscore)
16	**250**
12	**270**
16	**230**
17	**300**
10	**270**
14	**300**
11	**300**
6	**280**
17	**170**
16	**165**
11	**300**
13	**230**
14	**300**
17	280
14	120
16	270
12	300
13	270
14	260
17	300
18	300
19	300
10	300
15	280
14	145
17	280
14	270
25	300
14	270
18	260
14	300
12	290
16	260
15	280
16	300
14	270
13	300
10	210
14	290
15	220
15	290

Table 4.13: Pre-treatment mvc and levels of ER expression: (CD31 staining):

Non-responders shown in **bold** type.

Pre-treatment mvc	**Level of ER expression** (Histoscore)
16	**250**
12	**270**
18	**230**
14	**300**
9	**270**
15	**300**
15	**300**
8	**280**
18	**170**
17	**165**
11	**300**
11	**230**
20	**300**
17	280
16	120
13	270
13	300
14	270
16	260
9	300
12	300
18	300
12	300
15	280
16	145
21	280
16	270
22	300
15	270
17	260
16	300
17	290
20	260
14	280
20	300
13	270
14	300
15	210
14	290
13	220
10	290

5. Changes in proportion of tumour cells expressing vascular endothelial growth factor and tamoxifen treatment.

Table A5.1: Proportion of VEGF-positive tumour cells before and after treatment with tamoxifen: (Non-responders in **bold** type)

PRE-TREATMENT VEGF	POST -TREATMENT VEGF
12	**12**
11	**11**
12	**12**
12	**4**
12	**0**
11	**5**
12	**6**
12	**9**
12	**11**
12	**11**
7	**12**
1	**9**
12	12
9	9
12	12
12	12
12	12
12	12
12	7
11	9
12	6
12	0
12	5
12	7
12	6
12	4
11	9
12	10
12	1
12	9
12	7
12	7
7	2
12	6
12	4
12	4
12	5
0	8
3	9
9	11
7	9
3	6
0	2
11	12
11	12
2	8
7	11
1	11

Table A5.2: Relationship between proportion of VEGF-positive tumour cells and microvessel counts before treatment:

a) Non-responders

Pre-treatment mvc	Pre-treatment VEGF
12	11
12	12
12	12
13	12
14	12
16	7
16	1
18	12
19	12
19	11
21	12
21	12

b) Responders

Pre-treatment mvc	Pre-treatment VEGF
10	12
10	11
10	7
11	7
13	12
13	12
13	12
13	12
13	11
15	12
15	12
15	12
15	0
16	12
16	12
16	12
16	12
17	3
17	9
17	0
18	11
18	12
18	12
18	3
18	2
18	1
19	12
19	12
21	12
22	11
23	9
24	12
24	7
25	12
26	12
35	12

Table A5.3: Relationship between % VEGF-positive tumour cells and microvessel counts after treatment.

a) Non-responders

Post-treatment mvc	Post-treatment VEGF
19	12
20	11
20	12
15	4
24	0
15	5
19	6
25	9
17	11
18	11
17	12
27	9

b) Responders

Post-treatment mvc	Post-treatment VEGF
18	12
21	9
8	12
18	12
22	12
13	12
11	7
14	9
12	6
21	0
19	5
10	7
32	6
9	4
12	9
15	10
9	1
15	9
11	7
27	7
18	2
11	6
12	4
23	4
15	5
11	8
11	9
15	11
15	9
16	6
16	2
9	12
17	12
21	8
7	11
14	11

6. Changes in tumour cell proliferation during treatment with tamoxifen.

Table A6.1: Changes in MIB-1 expression during treatment with tamoxifen in non-responding tumours:

Pre-treatment MIB-1	Peri-treatment MIB-1	Post-treatment MIB-1
4	1	2
2	3	2
3	3	5
2	2	2
4	4	4
3	2	2
3	2	4
2		5
4	3	4
4	4	3
3	2	
3	3	3
2	3	2

Table A6.2: Changes in MIB-1 expression during treatment with tamoxifen in responding tumours:

Pre-treatment MIB-1	Peri-treatment MIB-1	Post-treatment MIB-1
3	3	2
4	3	3
3	2	3
2	2	2
2	2	
2		2
2	2	2
2	1	2
4	5	4
2	2	1
4	3	3
2	3	2
3	1	2
2		2
3	2	
5		
3	2	1
3	2	3
5	3	3
2	2	2
2	3	2
2	2	3
3		2
2	2	2
2	3	
2	3	2
3	3	3
2	2	2

Abstracts and Publications.

Abstracts:

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T.J. Anderson, L.P. Marson, C. Bellamy, K.K. Kurian, L. MacFArlane, J.M. Dixon, U. Chetty, W.R. Miller. Pathological features of breast cancer response to neoadjuvant treatment with the aromatase inhibitor letrozole.
Breast Cancer Research and Treatment 1998. **50 (3).** 305.

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L.P. Marson, W.R. Miller, J.M. Dixon. Angiogenesis and Breast Cancer.
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Accepted for publication in British Journal of Cancer.